

A COMPARISON OF OVARIAN FUNCTION IN JUVENILE AND ADULT EWES USING *IN VITRO* CULTURE AND PROTEOMICS

BY

MOHAMMED A YOUNES, M.Sc.

**THESIS SUBMITTED TO THE
UNIVERSITY OF WALES FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

JUNE 2008

**INSTITUTE OF BIOLOGY, ENVIRONMENTAL AND RURAL SCIENCES
ABERYSTWYTH UNIVERSITY
LLANBADARN CAMPUS
ABERYSTWYTH
CEREDIGION
SY23 3AL**

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed (candidate)

Date

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed (candidate)

Date

STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed (candidate)

Date

Acknowledgement

There are many people, too many to mention in person, who have helped me in various capacities throughout this project. I am indebted to all of the academic, technical and administrative staff and fellow postgraduate student, in the Institute of Rural Sciences, Aberystwyth University.

First and foremost, I must extend a special thank you to my supervisor, Dr. Neil Beck for his generous help and support, during my time in Aberystwyth. I am particularly grateful to Dr Basil Wolf and Dr. Malcolm Leitch (IBERS), for their expert statistical advice, my numerous silly questions. I would also like to extend my gratitude to Dr Jo Hamilton for her assistance in protein analysis. My thanks also go to Mr. Brian Davies for his expert technical assistance with the project and providing me with much needed additional support. Thanks must also be extended to. Thanks must also be extend to Bethan Miles for her helping my in prove writing of my thesis.

I also thank my brothers Dr Mohamed El-Hamli and Dr. Abdullah Ibrahim (University of Sebha) also to Yazan Mansour and Fisal Al-Bana for their generous helping during my study period. Special thanks to my wife and our children, Fatma, Heba, Hajer and Ahmed for providing me with an inspiring and conducive family atmosphere whilst in the UK. It's been a hard slog for both of us over the last two years, but we've made it in the end.

Last, but not least, my deepest gratitude is to my dad, my family members, and my friends, whom has unfailingly supported me through the highs and, more importantly, the lows.

**THIS THESIS IS DEDICATED TO
THE MEMORY OF MY MOTHER,
FATMA**

Summary

This work was carried out to compare the endocrine function of ovarian tissue isolated *in vitro*, in an identical environment between ewes and ewe lambs. Furthermore, to determine whether the differences in endocrine and reproductive function of ewes and ewe lambs are related to differences in the proteomics of corpora lutea, follicles and oocytes.

Oestradiol concentrations in tissue cultured in TCM-199 were similar for ewe and ewe lamb follicles collected post slaughter on day 9 to 12 of the oestrous cycle and cultured for different incubation times but increased with increase in follicular size. Oestradiol secretion was greater ($P<0.001$) for ewe and ewe lamb follicles cultured in media with FCS. Media progesterone concentrations were higher ($P<0.001$) for ewe than ewe lamb follicles. Progesterone in media and in follicular fluid was increased with increased follicles size. Ewe CL, collected on day 9 to 12 of the oestrous cycle, produced more progesterone than ewe lamb CL when cultured in TCM-199 with or without FCS, PVA, BSA. Proteomics indicated more large spots, in ewe follicular and CL tissue average gels compared with ewe lamb average gels. The protein spots were estimated to be between 45 to 97 kD, in both tissue and age groups, this range of molecular weight could have affected steroid hormone synthesis. (Chapter.3).

Ewe and ewe lamb follicles cultured with FSH and LH produced more oestradiol than without, furthermore, oestradiol concentration increased with follicle size. There was no difference in media oestradiol concentration between age groups after 24 h of culture. However, for follicles cultured for 2, 4 or 6 h, concentrations were greater after 4 and 6 h, in ewes than in ewe lambs. Overall ewe lamb follicles produced more progesterone ($P<0.001$) than ewe follicles when cultured with FSH and LH when cultured for 24 h, but no difference was observed after 2, 4 and 6 hours between ewes and ewe lambs. Overall ewe follicles produced more ($P<0.002$) progesterone than ewe lambs when cultured with different concentration of hCG although there was no difference between ages with respect to oestradiol concentrations. Ewe CL secreted more progesterone ($P<0.002$) than ewe lamb CL, when cultured for 0 or 24 h. Furthermore, tissue concentrations were greater in ewe CL than ewe lamb CL after incubation in TCM-199, TCM-199 plus BSA, TCM-199 plus FCS and TCM-199 plus PVA. Ewe lamb CL produced more progesterone than ewe CL in medium containing LH when cultured for 2, 4 and 6 hours, but ewes produced more progesterone than lambs when CL were cultured with different concentrations of hCG. Relative to untreated shells, the protein profiles of the ewe follicular shells treated with FSH and LH changed to a greater extent than that of the ewe lambs treated gels in both ages contained more protein spots than control gels. The largest spots were estimated to be between 30 and 97 kDa (Chapter.4).

There was no difference between age groups for follicles from ewes and ewe lambs treated with ovagen in oestradiol and progesterone concentrations observed after 2, 4, 6 and 8 h of incubation in TCM-199. However, treatment with ovagen plus hCG resulted in higher oestradiol and progesterone concentrations in the media from ewe follicles compared to ewe lambs. Furthermore, there were more protein spots in the range 30 to 66 kDa marker in gels from ewes treated with either ovagen or ovagen plus hCG than for ewe lambs (Chapter 5).

Lamb oocytes were smaller than ewe oocytes and developed to a lesser extent in culture. Furthermore, the addition of FCS to TCM-199 caused greater cytoplasmic and nuclear maturation than other media used in this experiment and ewe lamb oocytes have a similar 1D protein bands compared with ewe oocytes, but contained less protein (Chapter 6).

Contents

Acknowledgement.....	II
Summary	IV
Contents.....	V
Glossary.....	XI
Introduction	XIV
Chapter One	1
Review of Literature	1
1.1 Pre pubertal ovarian development.....	2
1.1.1 Fetal stage.....	3
1.1.1.1 Morphology of follicle development	6
1.2 Puberty in Sheep	7
1.2.1 Onset of puberty in ewe lambs.....	8
1.2.2 Factors affecting puberty in ewe lambs	9
1.2.2.1 Breed	10
1.2.2.2 Age	10
1.2.2.3 Nutrition	11
1.2.2.4 Photoperiod	12
1.2.2.5 Breeding season	13
1.2.2.6 Bodyweight	13
1.3 The Oestrous cycle	15
1.3.1 Pineal gland and melatonin	17
1.3.2 Follicular phase	19
1.3.3 Folliculogenesis.....	21
1.3.4 Factors controlling Folliculogenesis	24
1.3.4.1 Gonadotrophin releasing hormone (GnRH).....	24
1.3.4.2 Follicle Stimulating Hormone (FSH).....	25
1.3.4.3 Luteinising hormone (LH)	26
1.3.4.4 Progesterone	27
1.3.4.5 Growth hormone (GH).....	28
1.3.4.6 Growth factors.....	29
1.3.4.7 Prostaglandin F2 α	32
1.3.4.8 Oestrogen	34
1.3.4.9 Factors controlling ovulation	35
1.3.5 Follicular atresia.....	37
1.3.6 Corpus luteum (CL)	39
1.3.6.1 Formation and development of corpus luteum.....	39
1.3.6.1.1 Factors affecting luteal function.....	41
1.3.6.2 Luteolysis of corpus luteum	44
1.3.7 Controlling the oestrus cycle with CIDRs	46
1.3.8 Progesterone	47
1.3.9 Prostaglandin (PG)	48
1.3.10 Gonadotrophin Releasing Hormone GnRH	49
1.4 Differences in the oestrous cycles of ewes and ewe lambs.....	49
1.4.1 Differences in ovulation, fertilization, conception and lambing rate between ewe lambs and ewes	50

1.4.2 Hormonal profiles of young and adult female sheep during the oestrous cycle and early pregnancy	51
1.5 Ovarian response to hormonal stimulation during the oestrous cycle and early pregnancy in ewes and ewe lambs	54
1.5.1 The administration of exogenous hormone to improve reproduction performance in sheep	57
1.5.2 Maternal recognition of pregnancy	58
1.6 Ovarian function in vitro	59
1.6.1 In vitro studies of follicular function	60
1.6.2 Studies on luteal function in vitro	63
1.6.3 Oocyte maturation	65
1.6.3.1 Oocyte maturation in vitro	67
1.6.3.2 In vitro studies of oocytes from ewes and ewe lambs.....	69
1.7 Embryo development in sheep	73
1.8 Protein expression and identification in sheep ovarian tissue.....	75
Chapter Two.....	77
General Materials and Methods	77
2.1 Introduction	78
2.2 Animals	78
2.3 Management of animals	79
2.4 Collection of ovaries and preparation of tissue	79
2.4.1 Collection of follicles	79
2.4.2 Collection of CL.....	80
2.5 Hormone radioimmunoassay	81
2.5.1 Progesterone	81
2.5.2 Oestradiol-17 β assay	82
2.5.2.1 Assay procedure	83
2.6 Sample preparation for electrophoresis.....	83
2.6.1 Protein purification (precipitation).....	84
2.6.1.1 TriChloro Acetic acid CHCL3O 2 (TCA) in acetone	84
2.6.1.2 Perfect FOCUS™ Protein Purification kit.	85
2.6.2 Protein estimation.....	85
2.7 Electrophoresis	86
2.7.1 One dimensional SDS - PAGE gel.....	86
2.7.1.1 Mini sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE),	86
2.7.1.1.1 12% separating 7cm gels (12%T, 3.3%C):	86
2.7.1.1.2 4%Stacking gel: Notes	87
2.7.1.1.3.Running buffer	87
2.7.1.1.4 One dimension 7cm gels electrophoresis	87
2.7.1.2 Experion™ Pro260 (Experion system).....	88
2.7.1.2.1 Preparing the gel stain and the filtered gel.....	88
2.7.1.2. 2 Preparing the sample buffer	88
2.7.1.2.3 Preparing the samples and the Pro260 ladder	88
2.7.1.2.4 Priming the chip	89
2.7.1.2.5 Loading the samples and Pro260 ladder into the chip	89
2.7.1.2.6 Running the Pro260.....	89
2.7.2 Two dimensional SDS - PAGE gels	90

2.7.2.1 First Dimensional SDS - PAGE gels:	90
2.7.2.1.1 Separating gel buffer	90
2.7.2.1.2 Stacking gel buffer	90
2.7.2.1.3 Running buffer	90
2.7.2.1.4 C1 buffer (sample buffer).....	90
2.7.2.1.5 Equilibration buffer	90
2.7.2.1.6 Agarose buffer.....	91
2.7.2.1.7 Isoelectric focusing of protein sample:	91
2.7.2.2 Second dimension SDS-PAGE.gel running	92
2.7.2.3 Gel casting for 2D gel electrophoresis	92
2.7.3 Protein gel staining.....	93
2.7.3.1 Silver staining.....	93
2.7.3.2 Coomassie blue stain.....	94
2.7.4 Gel scanning and analyse	94
Chapter Three.....	95
A comparison of basal steroidogenesis function, <i>in vitro</i> and proteomics in follicles and corpora lutea from ewes and ewe lambs	95
3.1 Introduction	96
3.2 Materials and methods	97
3.2.1 Experiment 1: Comparison of oestradiol and progesterone concentrations in media and follicular fluid of follicles from ewes and ewe lambs cultured <i>in vitro</i>	97
3.2.1.1 Follicles cultured for 24 h in TCM-199	97
3.2.1.2 Follicular fluid from 24 h cultured follicles in TCM	98
3.2.1.3 Follicles cultured for 2, 4, 6 and 8 h in TCM.....	98
3.2.1.4 Follicles cultured for 24 h in TCM-199 with or without FCS	99
3.2.2 Experiment 2	99
3.2.2.1 Comparison of progesterone secretion from CL cultured <i>in vitro</i> from ewes and ewe lambs	99
3.2.2.2 Comparison of progesterone secretion from CL cultured in different media	100
3.2.3 Proteomics of follicular shells and CL from ewes and ewe lambs	101
3.2.3.1 Follicular shells protein determination.....	101
3.2.3.1.1 Sample preparation.....	101
3.2.3.1.2 Two dimension electrophoresis (2-DE)	102
3.2.3.2 Corpus luteum	102
3.2.3.2.1 Sample preparation.....	102
3.2.3.2.2 Two dimension electrophoresis (2-DE)	103
3.2.4 Statistical analysis	103
3.3 Results	105
3.3.1 Oestradiol and progesterone concentrations in the media from follicles cultured <i>in vitro</i> from mature ewes and ewe lambs	105
3.3.1.1 Oestradiol in media of follicles cultured in TCM-199.....	105
3.3.1.2 Oestradiol in follicular fluid.....	106
3.3.1.3 Follicles cultured for 2, 4, 6 and 8 h in TCM-199	107
3.3.1.4 Oestradiol in media from follicles cultured for 24 h in TCM-199 with FCS	108
3.3.1.5 Progesterone concentrations in media of follicles cultured in TCM-199	108
3.3.1.6 Progesterone concentration in follicular fluid.....	109

3.3.1.5 Progesterone concentration in the media of follicles cultured for 2, 4, 6 and 8 h in TCM-199.....	110
3.3.1.6 Progesterone concentration in media from follicles cultured for 24 h in TCM-199 with FCS.....	111
3.3.2 Corpora lutea progesterone concentration.....	112
3.3.2.1 CL cultured for 2 h in TCM-199.....	112
3.3.2.2 Progesterone concentrations in the media of CL quarter cultured in four different types of media.	113
3.3.2.3 Progesterone concentrations in CL tissue cultured in four different media	114
3.3.3 Protein electrophoresis analysis	115
3.3.3.1 Protein identification from follicular shells	115
3.3.3.2 Ewes and ewe lambs luteal tissue electrophoresis	119
3.4 Discussion	122
3.5 Conclusion.....	129
Chapter Four.....	131
A comparison of hormone stimulated follicular/luteal steroidogenesis <i>in vitro</i> , and follicular proteomics in ewes and ewe lambs	131
4. 1 Introduction	132
4. 2 Materials and methods	132
4. 2. 1 Experiment 1: To compare oestradiol and progesterone secretion in ewe and ewe lamb follicles cultured in vitro in TCM199 with different concentrations of gonadotrophins.....	132
4.2.1.1 Experimental 1a: Follicles cultured in one concentration of FSH/LH.....	132
4.2.1.2 Experiment 1b: Follicles cultured in different concentrations of FSH/LH	133
4.2.2 Experiment 2: To compare progesterone secretion in ewe and ewe lamb corpora lutea cultured in TCM-199 with different concentrations of LH or hCG	134
4.2.2.1 Experiment 2a: CL cultured in four different concentrations of LH for 24 hours.....	134
4.2.2.2 Experiment 2b: CL cultured in four different concentrations of LH for 2, 4 and 6 hours	135
4.2.2.3 Experiment 2c: CL cultured in four different concentrations of hCG for 2 and 4 hours	136
4 2.3 Proteomics of follicular shells.....	136
4.2.4 Statistical analysis	136
4.3 Results	137
4.3.1 Experiment 1a: Follicles cultured in TCM199 with or without FSH/LH for 24 h	137
4.3.1.1 Oestradiol	137
4.1.1.2 Progesterone	138
4. 3. 1. 2. Follicles cultured in TCM-199 with different ratios of FSH/LH for different incubation times	139
4. 3. 1. 2.1 Oestradiol concentrations in media from follicles cultured with FSH/LH.....	139
4.3.1.2.2 Progesterone concentrations in media from follicles cultured with FSH/LH	140

4.3.1.2.3 Oestradiol concentrations in media of follicles cultured with different concentrations of hCG.....	142
4.3.1.2.3 Progesterone in media of follicles cultured with different concentrations of hCG.....	144
4.3.2 CL cultured in LH	146
4.3.2.1 Progesterone concentrations in media from CL culture with four different concentrations of LH for 24 hours	146
4.3.2.2 Progesterone concentration in the tissue of CL cultured in different concentrations of LH for 24 hours.	146
4.3.2.3 CL cultured in four different concentrations of LH and cultured for 2, 4 and 6 hours.	147
4.3.3 CL cultured in four different concentrations of hCG for 2 and 4 hours.....	149
4.3.4 Proteomics of small medium and large follicles from ewe lambs and ewes incubated with FSH and LH.....	151
4.3.4.1 Ewes small follicular shells.....	151
4.3.4.2 Ewes medium follicular shells	154
4.3.4.3 Ewes large follicular shells	157
4.3.4.4 Ewe lambs small follicular shells.....	160
4.3.4.5 Ewe lambs medium follicular shells	163
4.3.4.6 Ewe lambs large follicular shells	166
4.4 Discussion	169
4.5. Conclusion.....	175
Chapter Five	176
<i>In vitro</i> steroidogenic function and proteomics of medium and large size follicles from ewes and ewe lambs treated with progesterone and gonadotrophins.....	176
5.1 Introduction	177
5.2 Materials and Methods.	178
5.2.1 Animals	178
5.2.2 Treatment 1: Ewes and ewe lambs treated with FSH (Ovagen) to induce folliculogenesis.	178
5.2.3 Treatment 2: Ewes and ewe lambs treated with ovagen and hCG to induce follicular growth and maturation.....	179
5.2.4. Blood samples	179
5.2.5 Follicle culture	180
5.2.6 Proteomics.....	180
5.2.7 Data analysis	181
5.3. Results	182
5.3.1 Treatment 1: Ewes and ewe lambs treated with Ovagen	182
5.3.1.1 Oestradiol concentrations in culture media of follicles.....	182
5.3.1.2 Treatment 1 Progesterone concentrations in culture media of follicles. ..	183
5.3.1.3 Blood progesterone levels for ovagen group	184
5.3.1.4. Treatment 1: Proteomics of follicular shell from ewes and ewe lambs treated with ovagen	185
5.3.2 Treatment.2: Ewes and ewe lambs treated with ovagen plus hCG	189
5.3.2.1 Oestradiol concentrations in media	189
5.3.2.2 Treatment 2 Progesterone concentrations in media	190
5.3.2.3 Blood progesterone levels for ovagen plus hCG group	191

5.3.2.4 Treatment.2 Proteomics of follicular shells from ewes and ewe lambs treated with ovagen plus hCG	192
5.4 Discussion	196
5.5 Conclusion.....	200
Chapter Six.....	201
A comparison of oocyte maturation <i>in vitro</i> in ewe lambs and ewes	201
6.1 Introduction	202
6.2 Materials and Methods	203
6.2.1 Oocytes collection	204
6.2.2 Oocyte maturation	204
6.2.3 Oocyte evaluation.....	206
6.2.4 Oocyte Staining	206
6.2.5 One dimension dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	207
6.2.5.1 Sample preparation.....	207
6.2.2.1 Running gel	208
6.3 Results	208
6.3.1 Oocytes maturation	208
6.3.1.1 Cytoplasm diameter measurement	209
6.3.1.2 Cumulus diameter measurement	209
6.3.1.3 Cytoplasm total surface area measurements	210
6.3.1.4 Cumulus total surface area measurements	211
6.3.2 Oocytes electrophoresis	212
6.3.2.1 One dimension dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	212
6.4 Discussion	213
6.5 Conclusion.....	216
Chapter 7	217
General Discussion.....	217
7.1 Conclusions	225
References	227
Appendices	260

Glossary

3 β -HSD	3 β -hydroxysteroid dehydrogenase
17 β -HSD	17 β -hydroxysteroid dehydrogenase
20 α -HSD	20 α -hydroxysteroid dehydrogenase
BMP-15	Bone morphogenetic protein-15
BSA	Bovine serum albumin
bST	Bovine somatotropin
BW	Body weight
cAMP	Cyclic adenosine 3',5'-monophosphate
Ca ⁺⁺	Calsum
CIDR	Controlled internal drug release
CL	<i>Corpus luteum / Corpora lutea</i>
Cx43	Connexin 43
CSF-1	Colony stimulating factor -1
CYP11A	Cytochrome P450 side-chain cleavage
CYP17	Cytochrome P450 17 α -hydroxylase 17,20 lyase
CYP19	Cytochrome P450 aromatase
DAG	2-diactlglycerol
DbcAMP	
DNA	Deoxyribonucleic acid
2D-PAGE	Two-dimensional poly acrlamide gel electrophoresis
DTT	Dithiothreitol
Dw	Distilled water
Ddw	Double distilled water
eCG	Equine cohronic gonadotrophin
ECM	Extra cellular Matrix
EDTA	Ethylenediaminetetraacetic aci
EGF	Epidermal growth factor
FCS	Fetal calf serum
FGF-1	Fibroblast growth factor-1 (acidic FGF)
FGFR	Fibroblast growth factors

FGF-1 mRNA	Fibroblast growth factor
FGFs	Fibroblast growth factors
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor(s)
FSH-RF	Follicle-stimulating hormone releasing factor?
GH	Growth hormone
GHr	Growth hormone receptor(s)
GJIC	Gap junction intracellular communication
GM-CSF	Granulocyte-macrophage colony stimulating factor
GnRH	Gonadotrophin-releasing hormone
GnRH-R	Gonadotrophin-releasing hormone receptor(s)
GPCR	Group protein-coupled receptor
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotrophin
IGF	Insulin-like growth factor(s)
IGF-R	Insulin-like growth factor receptors
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGFBP-1 to -6	Insulin-like growth factor binding protein(s)-1 to -6
IFN- γ	Interferon γ
IL-1	Interleukin-1
IVEP	In vitro embryo production
kDa	Kilo Dalton
LH	Luteinising hormone
LHR	Luteinising hormone receptor(s)
LIF	Leukaemia inhibitory factor
MI	Meiosis I
MII	Meiosis II
mRNA	Messenger ribonucleic acid(s)
MW	Molecular weight
PAGE	Polyacrilamide gel electrophoresis
P cream	Progesterone cream

PDGF	Platelet derived growth factor
PBS	Phosphate buffer Slain
p-FSH	Purified follicle stimulating hormone
PGE2	Prostaglandins
PGF _{2α}	Prostaglandin F _{2α}
PKC	Protein kinase C
PLC	P
PMH	Premamillary hypothalamic are
PMSG	Pregnant mares serum gonadotrophin
PVA	polyvinyl alcohol
rBST	Recombinant bovine somatotropin
RIA	Radioimmunoassay
TAE	Tris-Acetate EDTA
TCA	Trichloro acetic acid
TGF- α	Transforming growth factor- α
TGF- β	Transforming growth factor- β
TGF- β	Transforming growth factors- β
TCM	Tissue culture medium
TNF- α	Tissue necrosis factor
TSH	Thyroid-Stimulating hormone
VEGF	Vascular endothelial growth factor
v/v	Volume/volume
w/v	Weight/ volume
ZP	<i>Zona pellucida</i>

Introduction

Infertility is one of the most important problems that facing researchers in the area of animal production area. There are many factors affecting infertility including ovarian dysfunction. Cystic ovaries, for example, caused by failure of ovulation or the persistence the corpus luteum are common in some farm animals. Corpus luteum persistence may be caused by inadequate PGF_{α2} discharge. Ovulation failure is one of the factors that can often cause a decline in fertility in farm animals. In this case the follicle grows normally and reaches preovulatory size without rupture, becomes partially luteinized, and then regresses. Persistent or frequent oestrus in animals with a normal genital tract, possibly with increased uterine tone and clear mucoid vulval discharge is indicative of a follicular cyst, conversely a failure to show oestrus may be indicative of a luteal cyst. In sheep with polycystic ovaries there is an increased number of antral follicles (Forsdike et al., 2007), and the earliest stages of follicle development are abnormal (Maciel et al., 2004).

In addition to luteal persistence, inadequacy of luteal function is one of the factors causing infertility in ewes (Downing, 1980). Embryonic loss, associated with in inadequate luteal function, particularly in first stage of gestation is common in all species, and especially in young farm animals (Wilmut, 1986). Is well documented that 25-55% of all mammalian embryos are lost during early pregnancy and the majority of these losses appear to be caused by insufficient luteal function (Niswender et al., 1994). In sheep embryonic mortality has been shown to be a major cause of reproductive failure (Diskin and Sreenan, 1985; Sales and Ashworth, 1986). Reproductive performance is lower in young female sheep than adults, with ovulation rates being lower and embryo mortality rates higher in lambs compared with ewes. This is due to inadequate reproductive development. Progesterone concentration is lower in lambs compared with ewes during early pregnancy (Davies, 1988a, Davies and Beck, 1993,

Khan et al., 1999). Consequently, although pregnancy rates are similar at day 15 post mating they are significantly lower in lambs compared with ewes by day 30 (Beck et al., 1996b).

Moreover, embryo losses ranging from 16 to 76 % occur in progestagen PMSG treated ewe lambs, particularly in the first stage of pregnancy (Quirke et al, 1983; Hamra and Bryant, 1979). Quirke and Hanrahan (1977) concluded that, the potential of 8-16-cell ova from progestagen PMSG treated ewe lambs to develop to full term is less than half that of similar ova from mature ewes. However, reproductive function was improved in ewe lambs following progestagen-PMSG treatment, which had the effect in synchronizing oestrus and the majority of lambs were mated within 2-3 days of sponge removal compared with control animals. However, only 19 % of the treated animals lambed.

Conception and lambing rates are also normally lower in ewe lambs mated naturally compared with yearling and adult ewes (Forrest and Bichard, 1974, Gordon, 1967, Quirke et al., 1979). In general, fertility is more variable and lower in ewe lambs compared with mature sheep. Furthermore, ewe lambs that do not conceive directly often fail to return to service, this contributes to the decrease reproduction performance (Davies and Beck, 1992). Ewes bred as lambs have higher lifetime production rate compared with those bred as yearlings (Bowstead, 1930, Briggs, 1936, Hulet, 1969, Longrigg, 1961) Therefore, ewe lamb subfertility is a major factor affecting lifetime and flock productivity (Dyrmundsson, 1973a)

The challenge of using ewe lambs for breeding and production is therefore that ewe lambs have low levels of embryo survival, as embryo losses occur at higher rate during and after implantation in lambs compared with ewes. This may result from ewe lambs having abnormal

oocyte development and a lower oocyte maturation rate than adult ewes. Furthermore, the cytoplasm of juvenile oocytes is incapable of supporting normal development due to abnormal cytoplasmic protein synthesis during oocyte maturation. (Dyrmundsson, 1973a, Dyrmundsson, 1983, Quirke et al., 1983). All of which may be related to abnormal ovarian function.

Abnormal embryo development may result from abnormal LH pattern during oestrus and progesterone secretion during the luteal phase as concentrations of these hormones were higher in ewes compared with ewe lambs (Davies and Beck, 1993). Although, Quirke, (1981a) found no difference between ewes and ewe lambs in the size of the LH peak or ovulation rate in progesterone/PMSG treated sheep. Treatment with hCG on day 12 post-oestrus or pregnancy, increased plasma concentrations of progesterone and oestradiol in ewes. In ewe lambs, however, the increase in progesterone and oestradiol concentrations was significantly less (Khan et al., 2007). This study concluded luteal and follicular function is subnormal in ewe lambs compared with ewes, which would affect oocyte quality and embryo viability. However, the difference in plasma hormone concentration reported above may be caused by factors other than differences in luteal and follicular function. For example there may be caused by differences in metabolic clearance rather or the number of corpora lutea or follicles present on the ovaries of ewes and ewe lambs.

Therefore the aim of this project was to compare the endocrine function of ovarian tissue from ewes and ewe lambs, isolated *in vitro*, in an identical environment. Furthermore, to determine whether the differences in endocrine and reproductive function of ewes and ewe lambs are related to differences in the proteomics of corpora lutea, follicles and oocytes.

Chapter One

Review of Literature

1.1 Pre pubertal ovarian development

The pre-puberty stage is the period from birth until puberty. In young female sheep the ovaries normally increase in size, dependant on breed; the ovary has numerous active follicles in different sizes particularly in highly prolific breeds. The follicles continue to grow throughout life or at least until the reserve is exhausted. In this stage the lamb does not show oestrous behaviour or ovulation until puberty, although some pulsatile luteinising hormone (LH) secretion can be observed in ewe lambs at 11 weeks old, with follicle stimulating hormone (FSH) concentrations increasing from 3-11 weeks after birth (Foster and Karsch, 1975).

A number of experiments conducted using ewe lambs demonstrated that the numbers of antral follicles at birth are high and variable (Kennedy *et al.*, 1974, Land, 1970). However, using post-mortem observations every 4 weeks from birth to 24 weeks and at 33 weeks of age, that the number of antral follicles is elevated and constant at 4-8 weeks after birth and then decreased to relatively stable numbers by the time of the first ovulation (Gonzalez De Bulnes *et al.*, 2003, Kennedy *et al.*, 1974).

Generally, gonadotrophins control follicular development. Serum concentrations of LH and FSH were found to be higher in ewe lambs born between March and July than in ewe lambs born in August (Fitzgerald *et al.*, 1982). LH pulse frequency starts to decrease from week 5 to week 10 after birth and then its secretion is likely to be stable from week 10 to week 25 and then increased between 25 and 30 weeks of age. Furthermore, FSH mean concentrations did not differ during the actual period of sexual development of ewe lambs. However, in ewe lambs from 5 to 10 weeks old, the secretion of FSH was obviously pulsatile, even though the pulsatility was decreased or missing in adult animals (Rawlings and Churchill, 1990).

In ewe lambs the first increase in follicle number (at about 16 weeks of age) and diameter ($\geq 3\text{mm}$ in size) happens at the time when FSH pulsatility in the maturing ewe lambs has been shown to have declined (Bartlewski *et al.*, 2002). There is a second increase in follicle number which occurs at 24 weeks old. This increase may be attributed to an increase in LH pulse frequency (Rawlings and Churchill, 1990). Bartlewski, (1999b) and Gordon (1996) reported that the mean diameter of ovulatory follicles and ovulation rate at induced puberty in ewe lambs were similar to that at synchronised oestrus in ewes.

1.1.1 Fetal stage.

The formation, development and maturation of female gametes occurs during the fetal stage (Crisp, 1992). This is usually divided into 3 stages: (a) genital ridge formation; (b) indifferent gonad stage and (c) sexual differentiation (Peters, 1978). In sheep, oogenesis starts during the very early period of foetal life (ca. day 23-24 of gestation) with the arrival of 1,000-2,000 stem cells known as the primordial germ cells (PGCs), which move to their target in an amoeboid fashion (Webb *et al.*, 1999, Crisp, 1992, Wassarman and Albertini, 1994), starting from the yolk sac to the gonadal ridge via the dorsal mesentery of the hindgut (Webb *et al.*, 1999, Hirshfield, 1991, McLaren, 2000). The gonadal ridge, which will eventually develop into the ovaries (Van den Hurk *et al.*, 1997), is positioned ventrally on the mesonephros, which is the middle of the 3 pairs of renal organs of the fetus. Hence, PGCs are extra-gonadal in origin.

Around day 40 all of the germ cells in the foetal gonads are surrounded by somatic cells and enclosed into sex cords which are sited in the cortex (vandenHurk *et al.*, 1997, VandenHurk *et al.*, 1995). These germ cells then differentiate into oogonia that have proliferated via a chain of mitotic cycles where they may number ca. 600,000 (Crisp, 1992). At this stage, 70-90% of the

germ cells in fetal ovaries are mainly oogonia or isolated oocytes with a small number of primary follicles (Clark *et al.*, 1996, Smith *et al.*, 1997, Smith *et al.*, 1993a). Only after the completion of mitotic proliferation are the germ cells transformed into an oocyte, and this occurs via progression through the stages of meiotic prophase leptotene, zygotene, pachytene, and diplotene (Hirshfield, 1991a) that typically commence by day 75 in fetal ovaries and is usually completed by day 100 of pregnancy in sheep (Smith *et al.*, 1993a). Meiosis at this phase, however, is not completed and oocyte development is brought to a halt in the diplotene stage of the first meiotic division (Hirshfield, 1991, Van den Hurk *et al.*, 1997, Webb *et al.*, 1999, Bacharova, 1985). Therefore, the completion of meiosis will take place after ovulation (Hirshfield, 1991a). Meiosis is re-stimulated by the pre-ovulatory LH surge, progression characterised initially by the rupture of the germinal vesicle. This is followed by the condensation and reorganization of chromosomes in an equatorial position and finally by the emission of the first polar body (Crisp, 1992, Hirshfield, 1991a). In the metaphase of the second cell division meiosis will stop and only when the spermatozoid penetrates the oocyte does meiosis complete with the emission of the second polar body (Franchimont *et al.*, 1988).

The primordial follicle is the first stage in the development of the follicle. It results in a large reserve of quiescent primordial follicles (Hirshfield, 1991a). The primordial follicles start to appear following the isolation of the 'naked' oogonia. The isolated oogonia become surrounded by a layer of flattened follicular cells (known as Epitheliocytes). They are called 'naked' because they lack follicular cell investment and are connected as a syncytium via intercellular bridges (Crisp, 1992, Hirshfield, 1991a). The surrounding layer of flattened follicular cells is derived from the rete ovarii or the surface of the ovary (Webb *et al.*, 1999, Byskov, 1986).

By Day 100 in sheep the primary follicles containing one layer of cuboidal granulosa cells can be observed and by four months approximately 19% of the germ cell population are present in follicles with up to three layers of granulosa cells (Smith *et al.*, 1997). The primordial follicles, as explained above, contain a single layer of granulosa cells. These follicles rest on a basal lamina, surrounding an oocyte in the diplotene stage of meiosis (Crisp, 1992, Hirshfield, 1991a, Paton and Collins, 1992). The pre-granulosa cells go into a longstanding quiescence period. The number of primordial follicles in most mammalian species is usually more or less fixed in late foetal life or shortly after, in postnatal life. This fixed number is referred to as the primordial pool. From this primordial pool, follicles are recruited into the growing pool. This occurs during the reproductive life of the animal. After stimulation, the follicles start to develop from this pool, passing through the prenatal, antral, and preovulatory developmental stages. They are either then selected for ovulation during the final stage of growth or, the majority of them become atretic. Another process through which follicles are lost, beside meiosis, is the process of attrition. This process of attrition continues throughout early postnatal life. (Chun and Hsueh, 1998, Fortune, 1994, McNatty *et al.*, 1992a, Taymor, 1996).

The first primordial follicles in sheep appear on about day 75 of fetal life. By day 135 approximately 90% of germ cells become primordial follicles, 4% turn into growing follicles which may develop to 0.25-0.80 mm in diameter with the remainder representing isolated oocytes. The pre-granulosa cells encapsulate most of the oocytes. The size of the pool varies in the postnatal ovary, dependent on many factors such as age, species and breed, but is mainly dependent on age. A good example be seen in human ovaries where there are $0.6 - 2 \times 10^6$ oocytes at birth. This number regresses to $3 - 4 \times 10^5$ at menarche and by menopause only 100 - 200 atretic oocytes are present. The explanation for this is age, which has an effect on

fecundity that has led to the extensive use of pre-pubertal ovaries as a source of follicles because they are the source of the most valuable yield of follicles particularly at the primordial stage (Webb *et al.*, 1999). The size of the follicle pool is also variable even between genetically identical animals of the same age. (Baker, 1963, Gosden and Telfer, 1987, McNatty *et al.*, 1992a, Richard and Sirard, 1996, Smith *et al.*, 1997, Wallace *et al.*, 1989, Webb *et al.*, 1999).

The estimated number of primordial follicles in young ewes is 0.4×10^5 with significant variations between animals and breeds. Besides age and breed, nutrition is also considered as a factor which affects the size of the pool (Borwick *et al.*, 1995, Borwick *et al.*, 1994, Robinson, 1996, Rae *et al.*, 2001). Undernourishment in fetal or neonatal life dramatically reduces the concentration of oogonia and primordial follicles in the ovine ovary. The primordial follicle population is basically the result of the first stage of follicle development producing a large reserve of primordial follicles. Primordial follicles typically reside on the outer cortex of the ovary. After being formed, they become quiescent with the pre-granulosa cells showing little signs of bioactivity. Follicles with 2 or more layers of granulosa cells are apparent by day 135 of fetal life and most follicles remain at this dormant stage until later on in life.

1.1.1.1 Morphology of follicle development

The time it takes for the primordial follicle to develop to the ovulatory size in sheep is longer compared with laboratory species, about 180 days (Cahill and Mauleon, 1981, Campbell *et al.*, 1995b). In large domestic ruminants the cycle of follicle growth and development depends on the oestrous cycle and is comparable with other monovulatory animals (Campbell *et al.*, 1995a, Lahloukassi and Mariana, 1984, McNatty *et al.*, 1992b, Rajkumar *et al.*, 1992, Ravindra *et al.*, 1994a, Smith *et al.*, 1997).

Studies have been conducted using sheep (Cahill and Mauleon, 1980b) and cows (Scaramuzzi *et al.*, 1980) which indicate that each day about 3 and 6 follicles, respectively, grow from the pool of primordial follicles because they acquire a cuboidal layer of granulosa cells. They grow to be intermediary and then primary follicles. Nevertheless, it is likely that the precise number of follicles entering the growth phase depends on the pool of non-growing follicles (Krarup *et al.*, 1969). As a follicle continues to grow it attains several distinctive morphological forms namely as: a thick acellular *zona pellucida* (ZP) which surround the oocyte; the *theca interna* (steroidogenic cells); the *theca externa* (connective tissue cells); a vessel system; a basement membrane; and a fluid-filled antral cavity (Hirshfield, 1991a) .

1.2 Puberty in Sheep

Generally, puberty is defined as the first time reproduction becomes possible in young females. Most ewe lamb breeds reach puberty in their first year. At that stage the young ewes start showing some behavioral changes as a result of hormone levels in the blood, particularly gonadotrophic hormones. These hormones are released from the pituitary gland to affect the ovaries and uterus. Usually, the signs of oestrous behavior are not clear during the first oestrous cycle and in addition, oestrus may not necessarily be accompanied by ovulation (Dyrmundsson, 1983).

The majority of sheep breeds start their breeding season from August until December every year and lambing occurs from March until May. Most of the lambs which are born in March, April or May attain puberty in the autumn of the same year. However, there are some factors that affect this such as time of birth, breed, nutrition, photoperiod and live weight (Dyrmundsson, 1973b, Joubert, 1963). In addition, many genetic, physiological and

environmental factors affect the onset of puberty. The photoperiod generally is the most important factor controlling the onset of puberty in sheep (Foster and Yellon, 1985). Overall reproductive function is affected by an enormous range of sensory inputs, which release both to the internal and external environment (Ebling, 2005). The first successful ovulation in Polish Merino sheep occurs at approximately the 58 weeks old, while the Suffolk breed, which is born in the autumn, reach pubertal age during the anoestrus period; Wankowska *et al.*(2007) found that, reproductive cycles did not occur and puberty was delayed until the following breeding season at about one year old. Puberty could be defined by including both the requirement to ovulate and to allow insemination by the ram as well as three consecutive, luteal phases (Dyrmondsson, 1983, Foster *et al.*, 1989).

The transition into adulthood in the ewes is characterised by three pre-ovulatory gonadotrophin surges, only the last one being capable of producing fertilized ovum (Abecia and Zunga, 2001). Changes in tonic of LH secretion plays a key role in the endocrine proceedings which initiate ovulatory ovarian cycles in young female sheep (Foster *et al.*, 1986), the negative feed-back of oestradiol on puberty allows tonic LH secretion to increase. In fact, the onset of puberty and subsequent post-pubertal reproductive function are affected by an enormous number of sensory inputs relating to both internal and external environments (Ebling, 2005). The plasma progesterone levels during the oestrous cycle, however, shows little variance (Quirke *et al.*, 1979).

1.2.1 Onset of puberty in ewe lambs

All the components of the hypothalamic-pituitary-ovarian axis are in place to facilitate the expression of the oestrous cycle. The hypothalamus is the primary site of change during the

attainment of puberty. The maturation of the hypothalamus leads to a weakening of the negative feedback control of oestradiol and this is responsible for an increase in the frequency of released LH pulses. It is the increase in tonic LH pulses that is the main endocrine factor regulating the onset of puberty in ewe lambs as this accelerates the development of ovarian follicles, which in turn produce enough oestradiol to regulate behavioural oestrus and the pre-ovulatory surge of gonadotrophin (Kinder *et al.*, 1995). Beck and Davies (1994a) reported that at the onset of natural puberty the reproductive system of ewe lambs is comparatively undeveloped and that this leads to a negative effect on the fertility of ewe lambs. However, fertility will increase as the reproductive system matures during subsequent cycles.

There are numerous factors which influence the increase of the release of pulsatile LH and these include genotype, physiological and environmental factors, nutritional status and exogenous hormone treatment which affect the onset of puberty (Kinder *et al.*, 1995). Attainment of puberty is also affected by variations, in age and body weight between and within breeds. High fecundity breeds (such as the Suffolk) which breed early in the year usually attain puberty at the beginning of the breeding season, while those with low fecundity (Welsh Mountain) born at about the middle of the lambing season do not experience puberty until the middle of the following breeding season (Ward, 1980).

1.2.2 Factors affecting puberty in ewe lambs

Puberty in ewe lambs depends on the interaction of several factors: environmental factors such as photoperiod, breeding season, body weight, genetic factors and hormonal factors such as luteal function, oestradiol level and follicle maturation. However, genetic factors (Breed), environmental factors and their interaction are the important factors controlling sheep puberty.

All these factors work together with multiple interactions to enhance puberty action. Food availability is one of the main factors which affects ovulation rate (Braden, 1989, Nagatani *et al.*, 1998).

1.2.2.1 Breed

Genotype and its interaction with environmental factors play a key role in sexual development (Gordon, 1997, Land, 1978). Furthermore, genetic effects on puberty are related to environmental factors, such as the season of birth, and the plane of nutrition (Haresign, 1983). Genetic variation was found among individuals of the same breed (Quirke, 1977a). In this regard the first oestrus normally differs among breeds, as the heaviest breeds enter puberty later than the lighter breeds; for example, the onset of puberty in Rambouillet ewe lambs are later than those in Columbia or Targhee ewe lambs (Gaskins *et al.*, 2005). Crossbred females tend to achieve better reproductive performance than purebreds as the Romanov crosses attain puberty earlier than the Karakul breed (Boshoff *et al.*, 1975).

1.2.2.2 Age

One of the factors which affects the puberty of ewe lambs is age, which is variable between breeds, but differences in age at first ovulation can be small. For example, Finnish Landrace lambs reach puberty at the age of 250 ± 30 days and Corriedale ewe lambs reach puberty at about 243 days. While Suffolk ewe lambs reach puberty at 240 days (Yue *et al.*, 1996). Karagouniko ewe lambs born in December to January start their sexual activity at about 238 days compared with those born in early November, which reach puberty around 300 days of age. Most ewe lambs in this breed reach puberty from 224 to 270 days of age, during their first

breeding season (Valasi *et al.*, 2006), which is thought to be controlled by reaching a threshold maturity body weight prior to puberty (Moreno *et al.*, 2000, Quirke and Hanrahan, 1977).

In Suffolk ewe lambs, follicles increase in diameter prior to ovulation, as a response to an increase in the frequency of LH surge at about 32 weeks old. In addition, a gradual increase in the concentration of oestradiol was observed from 6 weeks until puberty was reached (Bartlewski *et al.*, 2006). In ewe lambs of the Columbia breed, the probability of pregnancy increased slightly with age at breeding. Additionally, in Rambouillet ewe lambs the probability of fertility increased quickly between 185 and 220 day of age, whereas in Polypay ewe lambs the weight at breeding did not influence the probability of pregnancy (Gaskins *et al.*, 2005).

A study conducted by Papachristoforou *et al.* (2000) noted that breed differences exist between the age at puberty and age at first parturition, which contrasts with the results of Quirke, *et al.* (1985) who found that the age at first ovulation was similar for Finn-Dorset, Suffolks, Finnish Landrace and Rambouillet breeds of lamb. Fertility and prolificacy are influenced by age as they increase with older ages at breeding (Gaskins *et al.*, 2005).

1.2.2.3 Nutrition

Nutrition is a chief factor influencing an animal's ability to reproduce (Webb *et al.*, 1999). Puberty in ewe lambs is mainly determined by genetic and environmental factors such as nutrition. Using a high plane of nutrition, ewe lambs reach puberty earlier than those on a low plane of nutrition and consequently, inadequate nutrition levels can delay the maturation of reproductive function (Robinson *et al.*, 2006). When ewe lambs were fed *ad-libitum* late in the breeding season until winter and early spring, they grew larger than the normal size for puberty without ovulation (Foster *et al.*, 1985). In British sheep, puberty may be delayed or advanced

by varying the plane of nutrition (Moreno *et al.*, 2000). In general, the interaction between reproduction and nutrition has major implications for reproductive performance in female sheep, whereby feeding can be used to control or delay puberty (Somchit *et al.*, 2007).

Generally, growth-restricted spring-born lambs reached puberty at a similar age and at equivalent live weights to the normally developed lambs. Furthermore, using a restricted nutrition plane leads to reduced LH secretion as a result of inadequate gonadotrophin releasing hormone (GnRH) release (Da Silva *et al.*, 2001). Several studies have mentioned that the level of nutrition, and therefore growth rate, during the pre-pubertal period can affect the timing of the onset of puberty (Adam and Findlay, 1997). T'Anson *et al.* (1997) suggested that the growth-restricted lamb is developmentally delayed in terms of its GnRH neurosecretory system.

1.2.2.4 Photoperiod

Photoperiod is the major environmental factor controlling seasonal reproduction, not only in sheep, but also in most mammals (Turek and Campbell, 1979). Exposure of ewe lambs to photoperiod signals of long days followed by short days is necessary for normal sexual maturation to reach puberty at the normal time, although it was found that photoperiodic sequence of long days before birth followed by short days after birth did not advance puberty (Foster and Yellon, 1985, Foster *et al.*, 1988). The exposure of lambs to photoperiod before birth does not influence the time to reach puberty after birth, nor does it affect reproductive development in ewe lambs (Sunderland *et al.*, 1995). Dyrmondsson (1983) concluded that ewe lambs born early in the season have a tendency to attain puberty earlier than those born later on. Many studies have been conducted into daylength as the main factor controlling the season

of sexual activity. Decreases in daylength controls the date of the onset of oestrus and increases in daylength controls the onset of anoestrus (Ducker and Bowman, 1970).

1.2.2.5 Breeding season

Lambs born in spring reach puberty in autumn of the same year, but lambs born in autumn reach puberty about four weeks later in the following season and are older by about four months compared with the spring-born lambs (Foster, 1981). In Damascus goats, females born in autumn reach puberty in the following autumn at the same time as young females born in February, but at an older age and heavier body weight (Yellon et al., 1992). Furthermore, mating ewe lambs in the third oestrus, at a greater age and weight, improves lambing performance compared to animals mated at puberty, even though ovulation rate and fertilization rate were similar at puberty and third oestrus (Beck et al., 1994b). Breeding seasons are shorter in young sheep and goats than in adults and oestrous cycles start later in the year in young animals than in adults (Quirke et al., 1983).

1.2.2.6 Bodyweight

Hare and Bryant (1985) reported that neither live weight nor condition score affected the fertility of ewe lambs mated at first, second or third oestrus while Dyrmondsson (1983) reported that heavier ewe lambs due to nutrition effects could favour an earlier onset of first oestrus. The total weight gain is different according to breed. An experiment conducted by Gaskins *et al.* (2005) using different ewe lamb breeds (Targhee, Columbia, Rambouillet and Polypay) concluded that the weights at breeding were similar for all breeds, but that the total weight gains from weaning to breeding were greater for the Targhee and Columbia than for Polypay and the Rambouillet being the latter gaining. This study indicated that breed, year, and

breed x year interaction were important effects, where the fertility was affected by type of birth and rearing. In addition there is no effect of the interaction between year and type of birth. Gaskins *et al.* (2005) concluded that heavier breeds of lamb (Columbia) have a higher fertility than lighter breeds (Rambouillet). The fertility rates were influenced by weight at breeding as well as by total weight gain from weaning to breeding. Higher fertility rates are usually associated in ewe lambs with heavier body weight (BW) (Dickerson and Laster, 1975, Dyrmondsson, 1981).

A study conducted by Laster *et al.* (1972.) said that BW at breeding did not influence the fertility of ewe lambs bred at an average age of 217d and that in general, older ages at breeding are connected with higher reproductive performance in ewe lambs (Dyrmondsson, 1973b, Laster *et al.*, 1972). Abecia and Zunga (2001) reported that the 28-week old Rasa Aragonesa ewe lambs that were born in spring and reached 75 % of their adult live weight (50 kg) exhibited sexual activity in response to progesterone treatment. Moreno *et al.* (2000) concluded that body weight is an important factor controlling puberty in both Mouflons and Manchega sheep, but that the minimum weight necessary to induce puberty can be quite variable between breeds of sheep.

Ewe lambs enter puberty during the breeding season where they reach approximately the optimum weight for each breed. For example, prolific Finnish Landrace reaches puberty at 32.7kg of body weight, Corriedale ewe lambs reach puberty at about 243 days and 28.8 kg, while Suffolk ewe lambs reach puberty at 41.17 ± 2.2 kg of body weight (Yue *et al.*, 1996) and Manchega ewe lambs when they weigh 69% of their mean mature weight Mouflons attain puberty when they weight 82% of their optimum adult weight (Moreno *et al.*, 2000). However,

physiological weight is one of the main factors controlling ewe lambs puberty during their first breeding season. In Mouflons and Manchega sheep, body weight appears to be an important factor governing the occurrence of puberty. Furthermore, the prevailing factor that determines the onset of puberty for the first breeding season, is the attainment with the heaviest mature body weight and fertility influenced by weight at breeding, where higher fertility rates of ewe lambs are generally related to heavier body weight and faster growth (Gaskins *et al.*, 2005, Moreno *et al.*, 2000). Ewe lamb breeds with the heaviest body weight reach puberty earlier than those born at the same time with a lighter body weight (Quirke *et al.*, 1979).

1.3 The Oestrous cycle

Sheep are seasonal breeders and their season runs from August to December. The ewe is polyoestrous and shows many oestrous cycles during this period. The oestrous cycle is repeated normally 17 days long and oestrus duration varies from 24 to 36 hours (Hafez, 1952), although cycle length varies amongst breeds. Luteolysis in cycling ewes normally occurs from three to four days before the next oestrus (Nephew *et al.*, 1991, Cardenas *et al.*, 1993). During spring and early summer months sheep exhibit inhibited activity referred to as the anestrous season (Pelletier *et al.*, 2000). The rate of fertility is different between breeds. Finnsheep and Dorset, which are the exception amongst British breeds, are classified as high fertility breeds and so are commonly used for spring breeding production systems. It was found that not seasonal breeders and are therefore the Romanov-sired ewes had greater fertility rate, longer length of seasonal fertility, increased prolificacy, as well as longevity compared with Finnsheep-sired ewes (Casas *et al.*, 2005).

At puberty the female reproductive system undergoes a well marked between hypothalamo-hypophyseal-utero-ovarian axes, functional rhythm known as the oestrous cycle which involves the involution. The oestrous cycle is divided into two phases, the follicular phase and the luteal phase. The follicular phase is relatively shorter than the luteal phase and takes about 2-3 days. At this time the animal's behaviour changes. The change from the follicular to the luteal phase is marked by ovulation (Figure. 1.1), although the transition from the follicular to the luteal phase is more complex (Heape, 1990). The ovulation process is principally controlled by gonadotrophin hormones (follicle stimulating hormone and luteinising hormone), which are secreted from the pituitary gland and the ovary steroid hormones, oestrogen and progesterone. The duration of the luteal phase is about 14-15 days from the end of ovulation and is governed by progesterone secretion. The progesterone is secreted entirely from the corpus luteum (Bjersing *et al.*, 1972).

It is also noted that the interval between the start of oestrus and the time of preovulatory LH discharge varies between and within breeds. The interval of LH surge tends to be greater in highly prolific sheep (18 hours) than in less prolific breeds (6-7 hours) (Gordon, 2004). Ewes show a special behaviour during oestrus. In the proceptivity period the ewe will be attracted to the presence of the ram, showing mutual sniffing and frequent urination and licking, followed by receptivity when the ewe stands to be mounted. The duration of the oestrous behaviour is about 30 to 48 h depending on the breed (Faber-Nys, 1987).

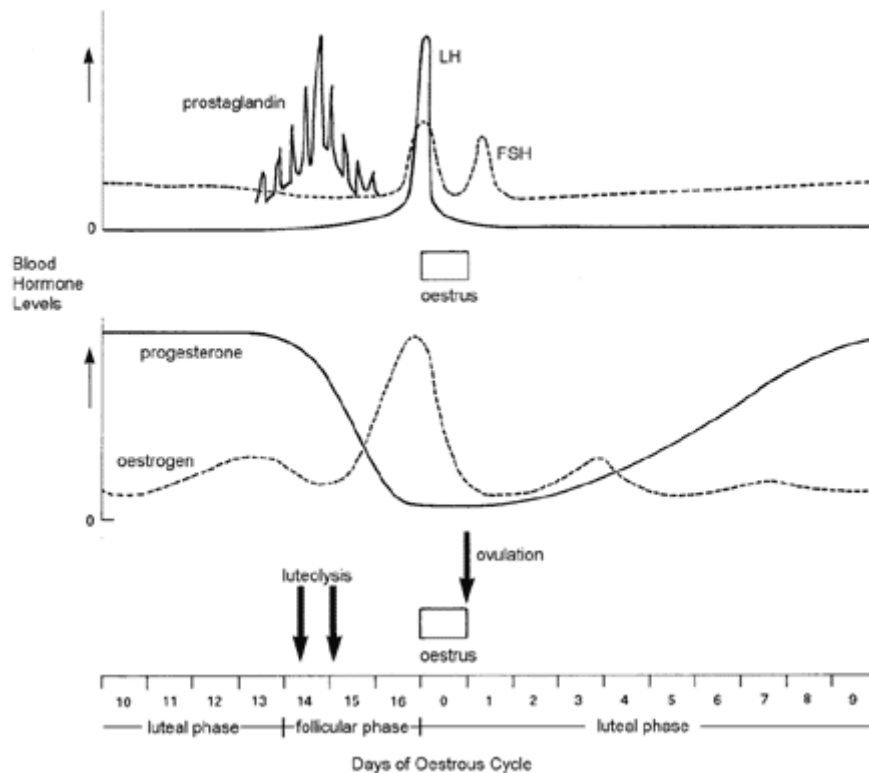


Figure.1.1 Changes in the levels of reproductive hormones in the blood during the oestrous cycle in the ewe. (From the internet web).

1.3.1 Pineal gland and melatonin

The pineal gland is located on the roof of the midbrain and it controls the overall seasonality of reproduction in sheep. The function of this gland is to respond to photoperiodic cues throughout the year by releasing the indolic hormone, melatonin (Bittman *et al.*, 1983, Legan and Karsch, 1980, Woodfill *et al.*, 1994). It is responsible for the transduction of neural inputs derived from light signals into chemical messengers in the form of a circadian rhythm of melatonin secretion that indicates the duration of darkness (Ghazi 1995). The transition from long days of summer to the short days of autumn are transduced by the pineal gland into an increase of night melatonin production (Foster *et al.*, 1985). Melatonin is released mainly during the hours of darkness, the amount of secretion reflecting the length of darkness (Karsch *et al.*, 1984). Therefore, the secretion of melatonin increases when the day length becomes

short (breeding season). This rhythmic release of melatonin regulates the activity of hypothalamo-hypophyseal activity and, in turn, seasonal changes in reproductive function.

A number of studies have reported that animals treated with melatonin have enhanced sexual puberty and maturity (Tortonese and Inskeep, 1992), including pre-pubertal buffalo-heifers and ewe lambs (El-Battawy, 1998, El-Battawy, 2006). In ewe lambs treated with melatonin after exposure to a long photoperiod, 80% had a successful pregnancy with a high lambing rate. These findings are in agreement with results reported by Rajkumar *et al.* (1992) who found that ewe lambs treated with melatonin on July achieved puberty and started cycling. In addition to these animals ovulating by week three, melatonin establishes its role in controlling puberty by increasing the activity of the GnRH pulse associated with the rise of gonadotrophin hormone secretion which induces oestrus (Robinson *et al.*, 1993). Furthermore, therapy with melatonin advanced the onset of the breeding season (Haresign, 1992). In the sheep, the preliminary hypothalamic area (PMH) is the main target for melatonin and its role in the breeding season. Melatonin is directly, influenced by the brain, to regulate seasonal changes in the pulsatile secretion of GnRH from the hypothalamus (Sliwowska *et al.*, 2004). Moreover, the neurons are responsive to melatonin, which transmits this photoperiodic information to both the GnRH system plus the reproductive neuroendocrine axis (Sliwowska *et al.*, 2004). Improved fertility and fecundity was shown in Assaf ewes after they were treated with melatonin in February, and melatonin can improve the number of ovulatory follicles reducing the atresia of medium and large follicles (Abecia *et al.*, 2007). Another possibility for melatonin action is via its receptors in the pars tuberalis and zona tuberalis of the pituitary gland, which stimulates FSH and LH secretion (Skinner and Robinson, 1995).

Additionally, studies have shown that the daily feeding of melatonin to sheep in the late afternoon under long photoperiods advances the onset of the breeding season (Arendt *et al.*, 1983, Kennaway *et al.*, 1982). Continuous application was also found to be effective (Nowak and Rodway, 1985) and this led to the development of slow releasing melatonin implants commercially known as regulin for application under field conditions (Keenaway *et al.*, 1987). These implants contain 18 mg melatonin and are placed subcutaneously at the base of the ear, where they are capable of releasing the hormone continuously over 70 days.

1.3.2 Follicular phase

The follicular phase begins with luteal regression and finishes with ovulation and primarily under hypothalamo-hypophyseal control. The main event in this phase is the LH secretion from the pituitary to elicit ovulation. This phase is characterised by a decrease in progesterone concentration at about day 15 of the cycle, after lysis of the corpus luteum (Carson *et al.*, 1979a, Hafez, 1952). During the follicular phase in sheep, the suggestion is that there are two (Brand and Jong, 1973) or three (Smeaton and Robertso.Ha, 1971) waves of follicle growth, each wave involves the recruitment of a cohort of follicles. The wave pattern in sheep is similar to that in cattle, and in the case of sheep, these waves are preceded by a rise in FSH concentrations which stimulate follicle wave appearance (Bartlewski *et al.*, 2000c, Evans *et al.*, 2002, Gibbons *et al.*, 1999). A selection of dominant follicles continue to grow and mature to the pre-ovulatory stage whilst the others in the wave undergo atresia. A complex regulatory system exists to determine which follicles are selected. At the start of the breeding season, the positive feedback effect of melatonin secretion from the pineal gland stimulates the hypothalamus. This allows the gonadotrophin-releasing hormone to be released from the

hypothalamus, and pass down through the hypophyseal portal tract to the anterior pituitary gland to release FSH and LH.

During oestrus, the dominant follicle (the ovulatory follicle, one follicle generally grows larger than the other follicles) inhibits the development of other follicles in both ovaries, whilst completing its growth (Fortune, 1994). On the other hand, some researchers concluded that the ovulatory follicle can delay the development of other follicles during the luteal phase (Ginther *et al.*, 1995, Ravindra *et al.*, 1994b, Rubianes *et al.*, 1997). Ovulatory follicles have more oestradiol and a high oestradiol: progesterone ratio in follicular fluid than other follicles in the same cohort (Evans *et al.*, 2000).

The removal of the dominant follicle increased FSH concentrations and encouraged the growth of the follicle with the next largest diameter (Evans, 2003). However, these findings differed from the results of Bartlewski *et al.* (1999b) and Flynn *et al.*, (2000) who suggested that a clear dominance does not happen in sheep as new follicle cohorts have been found to emerge at the same time as ovulatory follicles of the last wave and these follicles somehow developed and ovulated.

It is well documented that ovarian function is primarily regulated by the gonadotrophin follicle stimulating hormone (FSH), luteinising hormone (LH) and their respective receptors follicle stimulating hormone releasing hormone and luteinising hormone releasing hormone (FSHR and LHR). FSH stimulates folliculogenesis whereas LH is responsible for ovulation. It is also evident that the locally-produced factors, such as steroid hormones, peptides and growth factors have an essential modulatory role in follicular development for example, regulation of

the recruitment, selection and dominance of the follicle and finally ovulation (Fortune and Quirke, 1988). In sheep, insulin like growth factor (IGF-I) plays an important role compared to IGF-II. IGF-I augments the production of FSH and/or GH and particularly stimulates oestradiol and progesterone production. However, IGF-I function was different between the early regression of small and large follicles (Hastie and Haresign, 2006a, Khalid *et al.*, 2000).

1.3.3 Folliculogenesis

Folliculogenesis is a very complicated process, and is recognized by the period of growth of an ovarian follicle, through the different stages of development from the time that it leaves the primordial pool, which is produced during oogenesis, until the time that it leaves or becomes atretic. This process in domestic ruminants, as with many other mammalian species, is controlled by a complex system, which incorporates both established endocrine mechanisms involving the hypothalamic-adenohypophyseal-ovarian axis, as well as other intra-and extra-ovarian factors (Hastie *et al.*, 2004).

The primordial follicle forms during fetal life or soon after birth. During the life time of the female there is a series of complex interactions. The sheep folliculogenesis process takes about 180 days from primordial follicle to ovulatory follicle (> 5mm) with 2-3 follicles leaving the pool begin to grow every day (Richards, 1980). However, there are some morphological changes that occur in the follicular fluid during follicular development and the diameter increases regardless of whether the follicle will undergo atresia or continue to grow (Richards, 1980). In the sheep ovary, the antrum configuration occurs when the follicular diameter reaches 0.2-0.4mm. These processes can take 40 days in order to be ready for ovulation (Lussier *et al.*, 1987, Turnbull *et al.*, 1977).

In the ovary the average number of ovulatory follicles present at any time is mostly constant, excluding oestrus (Webb and Gauld, 1985). This is despite the secretion of FSH at a low level during the luteal phase (for about 4-5 days) and consequently a number of follicles develop (Campbell *et al.*, 1991a). However, the waves of ovulatory follicle development in animals are regulated by the oestrous cycle and inhibited by the process of ovulation. Ovulation or atresia is controlled by endocrine changes that occur during the follicular phase and the new ovulatory follicle is determined by as a response to the peak of FSH concentrations on day one (Campbell *et al.*, 1990a).

The female sheep ovary at this stage contains a large number of follicles (12,000- 86,000), which are surrounded by ≤ 2 layers of granulosa cells, and approximately 100- 400 large follicles which have ≥ 3 layers of granulosa cells. Six months is needed for primary follicles (0.06mm diameter) to develop to the preovulatory stage, and a further 130 days of follicular growth occurs to develop from the prenatal to the antral stage (from 0.06mm to 0.2mm). Another 34 to 43 days is required to reach the pre-ovulatory stage (Cahill and Mauleon, 1980a, Turnbull *et al.*, 1977).

When a follicle is recruited it continues to grow until ovulation or degeneration. The follicular growth and maturation represents a series of sequential subcellular and molecular transformations of various components of the follicle: the oocyte, granulosa and theca (Testart *et al.*, 1982). These are governed by several intra-ovarian factors, intrafollicular factors, and hormonal signals, which lead to the secretion of androgens and oestrogens (mainly oestradiol). The follicle growth involves hormonally-induced proliferation and differentiation of both theca and granulosa cells, leading ultimately to the increased ability of follicles to produce oestradiol

and to respond to gonadotrophins. Oestradiol production determines which follicle will gain LH receptors necessary for ovulation and luteinisation. The follicles failing to ovulate undergo atresia.

All healthy follicles, about 2 mm in diameter, are recruited and once selection has occurred recruitment is blocked. FSH plays a major role in the initiation of antrum formation. This gonadotropin stimulates granulosa cell mitosis and follicular fluid formation and also induces sensitivity to LH by increasing the number of LH receptors. The presence of LH receptors in both thecal and granulosa cells (Webb and England, 1982) increase their ability to secrete more oestradiol than non-ovulatory follicles. An increase in follicular size and the antral fluid concentrations of oestradiol, testosterone and progesterone (England *et al.*, 1999) are the characteristics of an ovulatory follicle.

The occurrence of follicular waves during the luteal phase, which reach the follicular size and steroidogenic competence of large antral follicles varies with specific periods of follicle growth Carllos *et al.* (1997) and Souza *et al.* (1996) reported that ovulatory follicles generally develop from larger antral follicles of about 5mm in diameter. Another study in contrast, reported that all follicles larger than 2mm are promoted into the ovulatory pool, or oestrogenic follicles (Tsonis *et al.*, 1984). During the luteal phase, oestradiol can sometimes be secreted in response to LH pulses, but this is uncommon (Baird *et al.*, 1976) as there is a decrease in the LH pulse frequency after day 3 of the luteal phase (Campbell *et al.*, 1990b, Hauger *et al.*, 1977). Follicular phase FSH levels show a decline as the dominant follicles secrete increasing amounts of oestradiol and inhibin (Baird and S., 1981., Baird *et al.*, 1991).

1.3.4 Factors controlling Folliculogenesis

Folliculogenesis is a complex event regulated by a complex interaction between intrafollicular steroids, extraovarian factors, hypothalamus –pituitary – ovarian feedback structure as well as growth factors. Follicle development pattern in sheep and cattle are similar with some morphological differences (Campbell *et al.*, 1995a, Driancourt *et al.*, 2001a, Findlay *et al.*, 1994, Lussier *et al.*, 1987, Roche, 1996, Turnbull *et al.*, 1977).

1.3.4.1 Gonadotrophin releasing hormone (GnRH)

GnRH is a decapeptide which is synthesized and stored in the medial basal hypothalamus. GnRH provides a hormonal link between the neural and endocrine system. In response to neural signals, pulses of GnRH are released into the hypophyseal portal system for the release of FSH and LH from the anterior pituitary (Hafez, 1952). In female sheep, more than 50% of GnRH hypothalamus-neurons are located in the preoptic area (Caldani *et al.*, 1995). However, progesterone is the principal negative feedback hormone on the GnRH pulsation generator, which reduces pulse frequency during the luteal phase (Goodman and Karsch, 1980, Karsch *et al.*, 1987). The negative feedback action of progesterone combined with oestradiol during the luteal phase has a negative effect on the GnRH pulse (Caraty and Skinner, 1999). GnRH is secreted from the lateral portion of the median eminence in the hypothalamus. It is contained exclusively in secretory granules. GnRH stimulates LH and FSH secretion (Baulieu and Kelly, 1990). During the follicular phase the pattern of GnRH secretion initially involves an increase in frequency and a decrease in amplitude, followed by a massive and sustained preovulatory GnRH surge (Karsch and Evans, 1996).

Generally GnRH, combined with GnRH receptors (GnRHR), play a role as a main regulator of reproduction (Fink, 1988). On the plasma membranes of the pituitary gland the gonadotroph

cells, via the binding of GnRH to its group protein-coupled receptor (GPCR), activate a range of intracellular signalling pathways that eventually control the synthesis and release of LH and FSH (Millar *et al.*, 2004, Neill, 2002.). Moreover, Millar *et al.* (2004) and Neill (2002) have found that in mammals, decapeptide GnRH-I and GnRH-II with their receptors (GnRHR) which increase the potential diversity of physiological action. In rats, there were it was demonstrated that oestradiol and progesterone in different physiological states controlled GnRH gene expression in the hypothalamus (Attardi *et al.*, 1997, Herbison, 1998, Thanky *et al.*, 2003).

1.3.4.2 Follicle Stimulating Hormone (FSH)

FSH is a glycoprotein of molecular weight of approximately 32 kD in sheep and whit a half life of 2-4 hours (McDonald, 1980). The major role of FSH is the maturation of ovarian follicles. It is one of the glycoprotein hormones, along with luteinising hormone (LH), thyroid-stimulating hormone (TSH), human chorionic gonadotrophin (hCG) and FSH releasing factor (FSH-RF) secreted from the hypothalamus. Each glycoprotein hormone consists of two noncovalently associated protein subunits, alpha (α ~ 15 kDa; the number of amino acids = 92) and beta (β ~ 23 kDa; the number of amino acids = 118) (Pierce and Pearson, 1981). β subunits differ for each glycoprotein hormone in mammals and are encoded by separate genes that are interspersed with conserved and variable sequences (Jameson and Hollenberg, 1993). In contrast, α subunits are encoded by a single gene and have identical amino acid sequences (Gharib *et al.*, 1990). This hormone is responsible for the growth and development of ovarian follicles to a mature or Graafian follicle. FSH, in the presence of LH, stimulates oestrogen production from the growing follicle. The synergistic action of both FSH and LH are responsible for the rapid growth of follicles after antrum formation (Kaulfuss et al., 1994). The

main role of FSH is the growth and maturation of ovarian antral follicles, prevention of atresia, induction of aromatase, granulosa cell proliferation, and induction of FSH and LH receptors in the ovary (Richards, 1994). During the oestrous cycle and anoestrus the mean FSH concentrations were similar, but throughout pregnancy FSH levels gradually decrease. During the oestrous cycle there are three waves of FSH concentration (Evans *et al.*, 2002) but during anoestrus no significant changes occurred during a 24 h period of sampling. The increase in tonic FSH secretion may be needed for the onset of the puberty process (Foster, 1981). The secretion of FSH is mainly controlled by the negative feedback of oestradiol and inhibin, which are found in high concentrations in follicular fluid (Baird and Smith, 1993). Inhibin, which controls oestradiol and progesterone, is the key endocrine feedback regulator of FSH. It appears that several factors produced from the hypothalamus, adenohypophysis and the ovary controls FSH production and secretion. FSH secretion is similar to that of LH, depending on the stage of the oestrous cycle. There is a difference in sheep in that FSH does not seem to follow a pulsatile pattern of secretion as happens with LH (Culler and Negrovilar, 1987). Padmanabhan *et al.* (1997). However, reported that the FSH secretion in sheep may be pulsatile in some respects.

1.3.4.3 Luteinising hormone (LH)

Luteinising hormone is a glycoprotein hormone with a molecular weight of 30 kD and a half life of 30 minutes in sheep (McDonald, 1980). The hormone is released from the anterior pituitary in a pulsatile pattern. According to Hauger *et al.* (1977) variation in pulsatile LH release occurred in sheep during the oestrous cycle and they found LH fluctuated markedly in a manner symptomatic of pulsatile discharges.

The function of this hormone is the stimulation of the final maturation of follicles which eventually culminate in the induction of ovulation and luteinization (McNeilly *et al.*, 1981). The regulation of hormone secretion is governed by hypothalamic control by via GnRH. FSH and LH are essential for the synthesis of oestrogen. These hormones also induce the final stages of oocyte maturation to metaphase II (Jainudeen *et al.*, 2000). LH is also firmly regulated by action of progesterone and oestradiol directly and indirectly by their effects on GnRH secretion. Progesterone has a negative effect on LH tonic release during the luteal phase of the oestrous cycle (Hauger *et al.*, 1977). Therefore, progesterone concentrations begin to decline after luteolysis, the LH concentrations begin to increase. The synergistic effect of progesterone and oestradiol are able to operate at two levels, with oestradiol priming both the GnRH pulse generator (Goodman *et al.*, 1981) and adenohypophysis (Girmus and Wise, 1991) to produce progesterone negative feedback. GnRH and LH secretion during the luteal phase are restricted by high concentrations of progesterone and low concentrations of oestradiol. The difference in the frequency of pulses is probably a result of fluctuating oestradiol concentrations observed throughout the luteal phase as well as variation in progesterone output from the CL during luteogenesis (Cupp *et al.*, 1995). During most of the luteal phase progesterone does inhibit tonic GnRH/LH secretion (Moenter *et al.*, 1991).

1.3.4.4 Progesterone

Progesterone is synthesized from cholesterol and is secreted from the ovary, testis, adrenal gland and the placenta. Pregnenolone, an intermediary product of progesterone synthesis, is converted to progesterone by non-endocrine tissues eg. endometrium, skin and mammary gland, by the action of 3 β -hydroxysteroid dehydrogenase (Slotin *et al.*, 1970) though the pathways of synthesis and catabolism of progesterone as shown in Fig.1.2. Progesterone is the

most important intermediate in the formation of corticoids, androgens and oestrogens. The major site of progesterone production is the corpus luteum and placenta. It is the hormone responsible for the establishment and maintenance of pregnancy in most mammalian species.

The length of the oestrous cycle depends particularly upon the life span of the corpus luteum, which actively secretes progesterone. Karsh *et al.* (1980) suggested that both oestradiol and progesterone in sufficient amounts are necessary for the secretion of LH and the expression of oestrous behaviour during the oestrous cycle. Hauger *et al.* (1977) showed that mean serum LH and progesterone concentration were inversely related, LH being highest during the early and late luteal phases and lowest in the mid-luteal phase of the oestrous cycle. They concluded that progesterone can inhibit tonic LH secretion in ewes and plays a major role in the secretion of LH during the ovine oestrous cycle. In non-pregnant sheep, plasma progesterone concentrations begin to rise from day 1, reaching a maximum concentration of progesterone on day 10 and 11 before declining from day 12 to the onset of oestrus when the values are below 1 ng/ml. If pregnancy is established, luteolysis is inhibited and the continued secretion of progesterone from the corpus luteum maintains uterine quiescence and provides an optimal environment for embryo survival and establishment of pregnancy (Vincent and Inskeep, 1986). Progesterone appears to play a major role in the regulation of uterine secretion of prostaglandin F₂ alpha from the uterus. It increases the lipid droplets in the ovine endometrium, a source of arachidonic acid, and also activates PG synthetase activity (Louis *et al.*, 1977).

1.3.4.5 Growth hormone (GH)

GH has long been recognized as the main hormone regulating postnatal somatic growth GH is synthesized and secreted from the adenohypophysis. GH is a metabolic hormone that has been

widely studied, and is essential for many metabolic processes such as general growth, milk and muscle fibre production. However, numerous actions of GH appear to be mediated via IGFs, especially IGF-I is not that GH receptors (GHR) have been localized to follicular cells in the ewe (Carlsson *et al.*, 1992). GH also modulates the synthesis of multiple mRNA species in mammalian tissue and is mitogenic for cellular stromal elements, like fibroblasts, adipocytes, macrophages and endothelial cells. GH plays an essential role in controlling metabolism and acts throughout the body to promote both balanced growth and cell differentiation (Kaiser *et al.*, 2001). It can also affect the ovary where it can increase the number of follicles that grow per wave (Gong *et al.*, 1991). A study was conducted by Joyce *et al.* (1998) which found that ewes treated with recombinant bovine somatotropin (rBST) produced a significantly more of 2-3 mm follicles. Gong *et al.* (1991) and Gong *et al.* (1993) reported that recombinant growth hormone (rGH) treatment in dairy heifers increases the number of small antral follicles. Many hormones influence the production and secretion of GH, as well as growth factors which are related to the reproductive cycle (Kaiser *et al.*, 2001).

1.3.4.6 Growth factors

In mammals, the IGF system consists of two ligands namely IGF-I and IGF-II, two cell-surface receptors IGF-R I and II (Froesch *et al.*, 1985) and at least six IGF binding proteins IGFBP-1 to 6 (Hwa *et al.*, 1999). IGF-I stimulates a key components in the steroidogenic pathway, causing an increase in progesterone secretion in ruminants (Liebermann *et al.*, 1996), humans (Villavicencio *et al.*, 2002) and pigs (Miller *et al.*, 2003) whilst IGF-II has been found as a progesterone synthesis *in vitro* (Sauerwein *et al.*, 1992). A number of peptide growth factors have also been implicated in the local regulation of granulosa cell function (Hsueh *et al.*, 1984). Among these, the involvement of IGF-I in the growth, development and differentiation

of ovarian follicles is well documented (Adashi *et al.*, 1985). In vitro studies showed that IGF-I enhances FSH actions and stimulates oestrogen and progesterone production in murine (Adashi *et al.*, 1985), bovine (Schams, 1987), porcine (Maruo *et al.*, 1988) and ovine (Monniaux and Pisselet, 1992) granulosa cells. Follicular growth is controlled largely by pituitary gonadotrophins and ovarian steroids (Richards, 1980) but Khalid and Haresign (1996) suggested an involvement of both IGF-I and IGFBP in ovine follicular development. Khalid *et al.* (2000) also reported a significantly higher IGF-I concentration in the oestrogenic follicles and suggested that the IGF-I system had a role as regulator of follicular activity that causes follicle development and maturation.

LH pulses are frequently increased as a result of increasing oestradiol secretion by oestrogenic follicles (Mcneilly *et al.*, 1982); subsequently, positive feedback occurs and obtains the preovulatory LH surge directly at the level of the adenohypophysis and ultimately to the hypothalamus by an increase in GnRH secretion. Moreover, Perks *et al.* (1995) reported that IGF-II gene expression at the beginning of the oestrous cycle in sheep was higher in large follicles compared with small follicles, whilst similar results were also shown in cattle (Perks *et al.*, 1999). High concentrations of IGF-II gene expression were found in small and healthy follicles; however, as follicles increased in diameter or became atretic the IGF-II expression declined (Hastie and Haresign, 2006a). High concentrations of IGF-II gene expression in small follicles in sheep suggest this ligand is probably significant for cell proliferation. Small follicles have a greater capacity to proliferate, as well as limited steroidogenic ability compared to large follicles (Monniaux, 1987). In addition to which, IGF-II is able to bind to LH receptors on thecal cells (Carson *et al.*, 1979b) and consequently stimulate the synthesis androgens.

In sheep, reduction in type I IGF-R mRNA expression occurs when follicles become atretic (Armstrong *et al.*, 2000, Hastie and Haresign, 2006b, Perks *et al.*, 1999). It is proposed that the decrease in type I IGF-R during atresia could be a reflection of hormone concentration, Type II IGF-R mRNA expression patterns are similar to IGF or type I IGF-R mRNA (Hastie *et al.*, 2004). The main difference between the expression patterns of both type I and type II IGF-R mRNA was the effect on follicle health conditions. Type II IGF-R mRNA concentrations, in general, were higher in atretic follicles during the oestrous cycle as compared with type I IGF-R mRNA. The reason for high concentrations of type II IGF-R mRNA may be attributed to tissue remodelling (Hastie and Haresign, 2006a). IGF-II only stimulates biological activity when bound to type I IGF-R, and not via type II IGF-R, in the ovary (Adashi *et al.*, 1990).

Growth of healthy antral follicles is commonly coupled with the reduction in gene expression of IGFBP-2, -3, -4, -5, and -6 (Armstrong *et al.*, 1998, Besnard *et al.*, 1996). Furthermore, during follicle growth in sheep the decrease in intra-follicular levels of IGFBP-2, -4 and -5 is attributed to the decrease in expression of their respective mRNA (Monget *et al.*, 1993). Therefore IGFBPs play a positive role in regulating IGF ligand bioavailability; however, there are some differences in gene expression and thus potentially the production of IGFBPs. These differences may be important in the local regulatory mechanism controlling follicle growth and atresia. There is a decrease in IGFBP-4 in mature follicles and this reduction is thought to be necessary for the maturation and dominance of the pre-ovulatory follicle (Rivera *et al.*, 2001, Rivera and Fortune, 2003). Berisha and Schams (2005) documented that in the preovulatory follicle, theca-interna and granulosa cells considerably increased vascular endothelial growth factor (VEGF) protein content in the total follicle tissue and in follicular fluid during the development stage of follicle growth. Moreover, during the final stage of follicle growth, the

expression of VEGF receptors unlike the VEGF protein is not regulated, in the theca-interna and granulosa cells of preovulatory follicle (Berisha et al., 2000, Pepper *et al.*, 1991).

The expression of fibroblast growth factor FGF-I mRNA during the final stage of follicular growth was comparatively greater and more stable in the theca-interna and follicular fluid than in the granulosa cells (Berisha *et al.*, 2004). FGF-2 mRNA was found to be greater in the theca interna of large follicles and lower in granulosa cells without a change in regulation. The transcription of FGFR in theca interna has an important increase coinciding with the start of oestradiol secretion ($E > 0.5-5$ ng/ml FF), unlike the absence of regulatory change in granulosa cells (Berisha and Schams, 2005) .

1.3.4.7 Prostaglandin F_{2α}

Prostaglandins are the cyclooxygenase products of arachidonic acid metabolism. Biosynthesis occurs in most cell types having the capacity of utilizing fatty acids. The proportions and types of prostaglandins vary within cell types and conditions. Among all the prostaglandins, only those of the E and F series are associated with reproduction. PGF_{2α} of uterine origin is particularly responsible for luteal regression in non-pregnant animals (Horton and Poyser, 1976). Prostaglandin F_{2α} plays a major role during parturition in most domestic species (Kindahl *et al.*, 1982.). A luteolytic dose of PGF_{2α} or its analogues causes luteolysis in animals with a mature corpus luteum: in sheep 5 days after oestrus (Acritopoulou and Haresign, 1980), in cows after 4-5 days (Rowson *et al.*, 1972.). Young corpora lutea are resistant to the effects of PGF_{2α}.

Both steroids and peptides are thought to be important factors for the synthesis and release of $\text{PGF}_{2\alpha}$. Progesterone increased the concentrations of PGF in curuncular and intercaruncular tissues and of 13-14 dihydro-15-keto prostaglandin $\text{F}_{2\alpha}$ (PGFM) in the jugular venous plasma but when oestradiol was superimposed on the progesterone-primed system, a marked increase of PGF was observed (Louis *et al.*, 1977). In a study conducted by Tasende *et al.* (2002) progesterone treatment particularly inhibited uterine oestradiol and progesterone receptors.

After production in the uterine tissue, $\text{PGF}_{2\alpha}$ is transported to the ovary either by a counter current transfer mechanism involving the uterine vein and ovarian artery (McCracken *et al.*, 1972, McCracken *et al.*, 1971) or by the lymphatic system (Heap *et al.*, 1985). The mode of action of $\text{PGF}_{\alpha 2}$ in bringing about luteolysis is still not fully understood. Reduced blood flow in the utero-ovarian vein (Pharriss *et al.*, 1970) or a direct action on the cells of corpus luteum (McCracken *et al.*, 1972) might be responsible for the luteolytic effect. It is bound by the receptors of large luteal cells, which results in an immediate fall in progesterone secretion. Pate and Condon (1989) suggested that $\text{PGF}_{2\alpha}$ prevents lipoprotein cholesterol being utilised for steroidogenesis. The large luteal cells as well as the posterior pituitary also produce oxytocin for luteolysis (Flint *et al.*, 1990, Leymarie and Martal., 1993, Rogers *et al.*, 1990). Flint *et al.* (1990) reported that an injection of oxytocin has been shown to increase uterine $\text{PGF}_{2\alpha}$ secretion. Both oestradiol and IGF are strong stimulators of oxytocin through an increase in uterine luminal epithelial oxytocin receptors (Schams, 1987, Zhang *et al.*, 1992). Progesterone plays the role of inhibiting oxytocin receptors, and thus inhibits $\text{PGF}_{2\alpha}$ release.

1.3.4.8 Oestrogen

The ovarian follicles are responsible for the secretion of oestrogens, under the control of the pituitary gonadotrophins. The theca interna cells synthesise androgens from cholesterol as shown in Fig 1.2, whereas the granulosa cells enzymatically aromatize androgens to oestrogen. The major ovarian oestrogen secreted is Oestradiol 17β (McCracken *et al.*, 1971). Secretion fluctuates during the cycle with the highest peak during the luteal phase and on the day of oestrus (Scaramuzzi and Land, 1978). Cox *et al.* (1973) showed that oestradiol 17β increase on about day 11 to 12 in non-pregnant ewes, which is before the first peak of PGF $_{2\alpha}$ on day 13, and then declines before a major rise at pro-oestrus. They concluded that oestradiol is necessary for complete luteolysis. Suzuki and Tamaoki (1980) reported that serum oestradiol decreased dramatically immediately after ovulation as a consequence of the shortage of aromatizable androgens for ovarian aromatase, due to the diminished activity of 17 α -hydroxylase and C-17-C-20 lyase. Hixon and Flint (1987) found that a luteolytic dose of oestradiol in sheep on day 9 and 10 of the cycle resulted in an episodic release of PGF $_{2\alpha}$ and a decline in plasma progesterone. They suggested the following sequence of events during oestrogen-induced luteolysis: a) induction of oxytocin receptors b) increased turnover of phosphoinositides c) onset of episodic secretion of PGF $_{2\alpha}$ and functional luteolysis. It has been proposed that progesterone and oestradiol regulate the endometrial release of PGF $_{2\alpha}$ by affecting both the activity of phospholipase C and its associated second messenger (cAMP) responsive pathways that may regulate production of PGF $_{2\alpha}$ (Raw and Silvia, 1991).

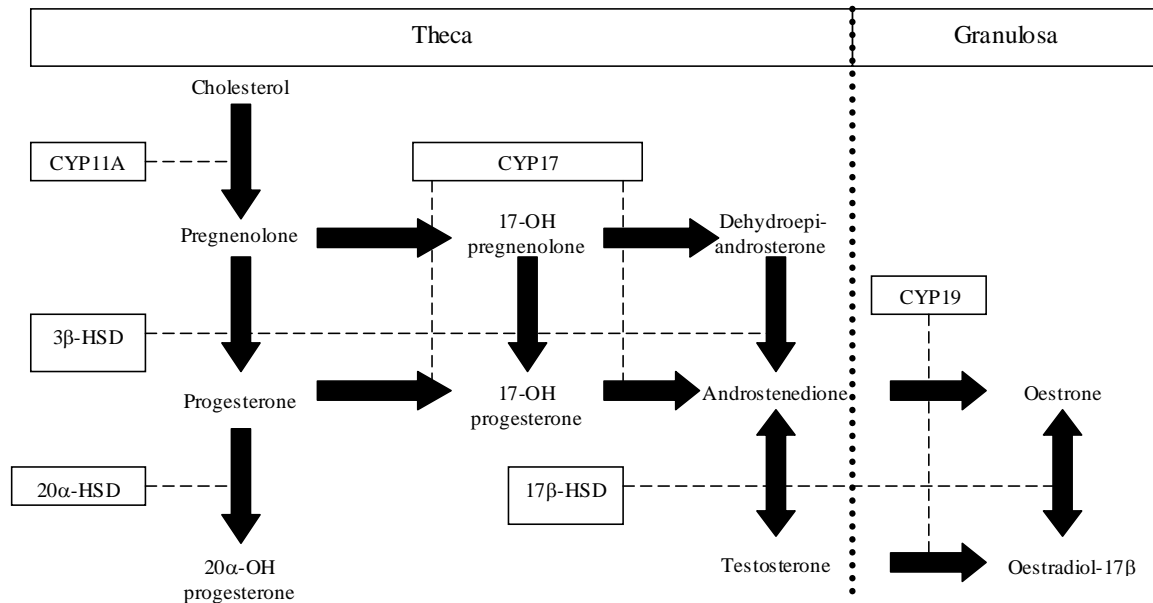


Figure. 1.2 Pathways of synthesis and catabolism of steroid hormones in ovarian somatic cells. Adapted from Strauss & Penning (1999). Enzymes involved in catalysing each step are shown in boxes. (CYP11A: cytochrome P450 side chain cleavage; CYP17: cytochrome P450 17α-hydroxylase 17, 20 lyase; CYP19: cytochrome P450 aromatase; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; 20α-HSD: 20α-hydroxysteroid dehydrogenase).

1.3.4.9 Factors controlling ovulation

Ovulation is a distinct biological process that requires the rupture of healthy tissue at the surface of the ovary. Various theories have been put forward to describe the mechanism of ovulation, namely the smooth muscle theory, pressure theory and proteolytic enzyme theory. In addition, the site of ovulation in the ewe is independent of the position of the CL of the previous oestrous cycle, which is unique relative to other species (Hafez, 1993).

At oestrus there is a release of LH when the mature follicle increases in diameter and finally ruptures with the release of the egg into the fallopian tube. The cells remaining from the

ruptured follicles proliferate and form the corpus luteum, which dominates the luteal phase of the cycle. The exact mechanism of ovulation is unclear, but previous work suggests that leukotriens may be important regulators (Reich *et al.*, 1983, Yoshimura *et al.*, 1991). In the absence of an LH surge and therefore ovulation, the pre-ovulatory follicles form follicular cysts (Freeman *et al.*, 1970).

A study conducted using rats, showed that the process of mammalian ovulation may be similar to an inflammatory reaction, with inflammatory-like changes occurring firstly in the theca interna and granulosa layers of the follicle in response to gonadotrophin stimulation of the luteinisation process (Espey and Lipner, 1994, Richards *et al.*, 2002). As a result, follicles could rupture due to tissue remodelling that is characteristic of stimulating reactions. This would lead to recruitment of thecal fibroblasts and loosening of the connective tissue in the follicle wall (Espey, 1994). Weakening and degrading of the extracellular matrix (ECM), within the theca layers and tunica albuginea especially, is essential in order for follicle rupture to occur (Richards *et al.*, 2002).

In most breeds of sheep, only one to three ovulations are formed at any one oestrous period and ovulation generally occurs at 24 hours after the LH surge (Cumming *et al.*, 1973). Mature follicles are the key necessity for ovulation and they must acquire sufficient concentration of LH receptors (Espey and Lipner, 1994, Richards and Hedin, 1988) so that the preovulatory LH surge can apply its effects. The preovulatory gonadotrophin surge then initiates a cascade of events that culminates in the exclusion of oocytes from the ruptured Graafian follicle. By now it is clear that LH is not the only hormone involved in the ovulatory process. In different species, several other hormones including FSH (Schenken *et al.*, 1984), GnRH (Eckholm *et al.*,

1981), prolactin (Piquette *et al.*, 1991), prostaglandins (Murdoch *et al.*, 1981), neuropeptide Y (Jorgensen *et al.*, 1991) and oxytocin (Wathes *et al.*, 1986), have been shown to participate in ovulation, even though this list is not comprehensive. Prostaglandins (PGE₂ and PGF_{2α}) are involved in the ovulatory process since they are produced by the follicle in response to the preovulatory LH surge (Murdoch *et al.*, 1981). This is supported by evidence that ovulation (although not luteinisation and consequent progesterone production) was blocked in sheep (Murdoch and Dunn, 1983) and rats (Armstrong and Grinwich, 1972) by the administration of indomethacin, a prostaglandin synthetase inhibitor.

On the onset of ovulation oestradiol levels are high but progesterone levels are low. Nevertheless, as oestradiol levels start to decrease rapidly, at the same time there is also a rapid increase in progesterone production (Espey and Lipner, 1994). By blocking progesterone synthesis using 3β-hydroxysteroid dehydrogenase/Δ⁵,Δ⁴ isomerase (3β-HSD) inhibitors, the ovulation rate is very inhibited, even though this inhibition can be overcome using exogenous progesterone (Espey, 1994). It was initially thought that oestradiol-17β was essential for ovulation, given that blocking ovulation by the use of amino glutethimide (which inhibits steroid synthesis) could be overcome by using exogenous oestradiol-17β or testosterone (Ying and Greep, 1971).

1.3.5 Follicular atresia

Follicles that fail to reach the final stage (maturation and ovulation) are called atretic and about > 99% of the ewe follicles end by atresia (Hsueh *et al.*, 1994, Jolly *et al.*, 1997a). Atresia is an apoptotic or a regulated structure of programmed cell death. Atresia of follicles is characterized by an unexpected and extensive death of the granulosa cells (Byskov, 1978, Byskov, 1979). It

is an irretrievable procedure that can occur at any period of development (Crisp, 1992, Greenwald and Roy, 1994) even though it is evident that atresia is not regularly prevalent during all stages of follicular development. Some physiological changes that happen during the atresia stage include a reduction in granulosa cell aromatisation, with the resulting loss in aromatase action (Huet *et al.*, 1997). Due to these consequences there is a shift in the oestrogen to progesterone ratio with a common decrease in oestrogen synthesis associated with a rise in testosterone and progesterone production (Hsueh *et al.*, 1994). In addition there is also a decline in the responsivity of the granulosa cells to gonadotrophin hormones, as it is clear from the effects on follicular synthesis of cAMP (Jolly *et al.*, 1997b). Evidence suggests that follicular atresia occurs by apoptosis rather than necrosis (Hurwitz *et al.*, 1996, Chun and Hsueh, 1998, Tilly *et al.*, 1997, Billig *et al.*, 1996, Hurwitz and Adashi, 1992, Driancourt *et al.*, 1998). In reality, necrosis seems to be a result of cell damage or other trauma (Hsueh *et al.*, 1994). Indeed, the rule of apoptosis and ovarian follicle atresia is a complex system incorporating a variety of hormonal mechanisms, also a large number of intra-ovarian factors and second messenger systems (Hsu and Hsueh, 1997, Kaipia and Hsueh, 1997, Tilly, 1996).

Morphologically the pattern of atresia seems to start with the formation of a few necrotic granulosa cells. The degeneration of many granulosa cells is observed in the granulosa layer within 24 hours, only few remnants of the granulosa cells or oocyte can be found after a few days (Osman, 1985, Hirshfield, 1991). Thecal cells can be observed during hypertrophy, after the atretic follicle has changed into a unit of secondary interstitial tissue. There are some classical indications in pre-antral and antral follicles due to atresia; these indications are granulosa cell senescence and sloughing of granulosa cells into the antrum; oocyte reduction and death; deterioration of the basement membrane; formation of pyknotic nuclei; shortening

of thecal cells; appearance of Call-Exner bodies; loss of the capillary network; and a reduction in the proportion of mitotic bodies (Marion *et al.*, 1968, Paton and Collins, 1992). To enhance ovulation, follicles must basically be prevented from following the atretic process (Hirshfield and Midgley, 1978).

IGFBP-5 may have an important role in the inhibition of IGFBP-4 degradation, as it is likely that IGFBP-4 proteolysis is necessary for follicle maturation, and degradation of IGFBP-4 is possibly important for atresia procedure (Fowlkes *et al.*, 1997, Hastie and Haresign, 2006b). Sheep follicle atresia is associated with the decrease in gene expression of IGFBP-2, -3, -4, and -6 in all sizes of follicles. An increase in IGFBP-2, -4, and 5 concentrations in follicular fluid of atretic follicles is proposed to occur as a result of a decline in the proteolysis of IGFBPs. Follicles with relatively higher IGFBP-5 gene expression develop to an advanced state of atresia.

During the advanced stage of atresia the IGFBP-4 proteolysis decreases as the result of IGFBP-5, whereas high levels of IGFBP-5 gene expression during advanced stages of atresia increase the inhibition (Fowlkes *et al.*, 1997). Furthermore, IGFBP-6 may inhibit IGFBP-4 degradation as high levels of IGFBP-6 mRNA are present in both small early follicles and large atretic follicles. This implies that IGFBP-6 is necessary in promoting the atresia of follicles <2mm diameter, via its effect on IGFBP-4 proteolysis (Hastie and Haresign, 2006b).

1.3.6 Corpus luteum (CL)

1.3.6.1 Formation and development of corpus luteum

The preovulatory surge of gonadotrophins induces ovulation and the differentiation of residual follicular cells which form the corpus luteum and begin to produce progesterone at a high rate.

Formation of the CL is initiated by a series of morphological and biochemical changes in the cells of the theca interna and the granulosa cells of the preovulatory follicle. These changes are termed luteinisation and occur after the preovulatory LH surge. The CL is a heterologous tissue consisting of endothelial cells, steroidogenic large luteal cells and small luteal cells as well as fibroblasts, smooth muscle cells and immune cells (Reynolds *et al.*, 1994). In a complex tissue, the various cell types must interact to ensure normal growth and development. Tissue growth depends on the growth of new blood vessels (angiogenesis) and the establishment of a functional blood supply. Blood flow to where decreases shortly after ovulation and then gradually increases afterwards in parallel with the increase in CL volume and plasma progesterone concentration from days 2-5 and also with increasing angiogenesis (Acosta *et al.*, 2003). This increase reflects normal luteal development and underlines the importance of angiogenesis. In the mature CL nearly every parenchymal cell is in contact with one or more capillaries (Redmer and Reynolds, 1996). The CL is one of the few adult tissues that exhibits a regular period of growth (angiogenesis), function and regression.

The theca- and granulosa-derived luteal cells give rise to two distinct types of luteal cells that differ morphologically and physiologically. The granulosa-derived cells are the large luteal cells, whereas the thecal cells give rise to small luteal cells. In ewes, the ovulatory follicular tissue that weighs ~ 40 mg, develops into a corpus luteum that weighs 600-700 mg in just a few days and the proliferation of cells in the developing CL results in a mitotic rate that is equal to that of a rapidly growing tumour (Jablonka-Shariff *et al.*, 1993).

1.3.6.1.1 Factors affecting luteal function

Corpus luteum function is governed by various factors including LH, which may activate cAMP, protein kinases or calcium. LH has a stimulatory effect on progesterone synthesis (Davies and Rueda, 2002, Grazul-Bilska *et al.*, 2001b). Milvae *et al.* (1996) reported that LH stimulates progesterone secretion through increasing cAMP concentration in luteal cells. LH also has an extra function in the CL, which is the stimulation of Cx43 mRNA expression in ovine luteal cells (Borowczyk *et al.*, 2007). The factors regulating proliferation of small luteal cells (SLC) and fibroblast may involve fibroblast growth factors (Grazulbilska *et al.*, 1996), growth hormone (Juengel *et al.*, 1996) and LH (Grazul-Bilska *et al.*, 1995). The vascular endothelial cells are probably regulated by vascular endothelial growth factor (Behrman *et al.*, 1971). The primary luteotropic hormones which support the development and function of the CL are LH and IGF.

The main function of CL is the production of progesterone. The synthesis of progesterone is derived from cholesterol through a steroidogenic pathway. The hormones that support the growth and/or function of the CL are termed luteotropic hormones and include LH, GH, prolactin, IGF-I, oxytocin, PGE-2 and PGI-2 depending on the species. LH receptors are located in the small luteal cell and progesterone production by small cells are LH dependent (Niswender *et al.*, 2000). Growth hormone receptors are located in the large luteal cells; these cells are responsible for 80% of total progesterone by the CL (Niswender *et al.*, 1985). Growth hormone has been indicated to stimulate progesterone and oxytocin secretion by bovine CL *in vitro* and support luteal development (Lucy *et al.*, 1994).

The corpus luteum is the main source of progesterone, which is essential for the maintenance of pregnancy in mammals. It secretes the largest concentration of progesterone in the mid-

luteal phase, which is day 10 of oestrus in sheep (Borowczyk *et al.*, 2007). This gland is a complex heterogeneous tissue consisting of many steroidogenic and nonsteroidogenic cells, where gap junctional intracellular communication (GJIC) plays the chief role in transferring nutrients, ions and regulatory molecules between adjunct cells, which appear in the ovine corpus luteum 48 h after ovulation. In addition, they regulate and coordinate cellular and tissue function in luteal tissue (Grazul-Bilska *et al.*, 1997). Borowczyk *et al.* (2007) demonstrated that there is a positive relationship between progesterone secretion and Cx 43 mRNA expression and between Cx43 mRNA expression and the GJIC of luteal cells. These findings indicate that the GJIC structure and function are involved in the regulation of luteal steroidogenesis and progesterone secretion. In addition, Cx43 governed the regulation of the secretion of adrenal cells (Oyoyo *et al.*, 1997).

LH and dbcAMP have a stimulatory effect on progesterone secretion during early luteal development by increasing the rate of gap junction intracellular communication (GJIC) between luteal cells and increasing progesterone secretion during the oestrous period (Grazul-Bilska *et al.*, 1997). Whereas progesterone production from CL depends on the stage of luteal development and LH and dbcAMP effects, which is confirmed by increased Cx43 mRNA expression during the early and mid-luteal period of the oestrous cycle after treatment with LH and dbcAMP (Borowczyk *et al.*, 2007). Moreover, ewes injected with a bolus of exogenous oLH on day 3 or day 11 of the luteal stage during the breeding season exhibited significantly elevated plasma progesterone concentrations (Bramley *et al.*, 2005). The extended luteal phase associated with higher plasma prolactin concentrations may have a role in luteal phase length, but the pharmacological elevation of prolactin failed to prevent luteolysis induced by $\text{PGF}_{\alpha 2}$ (Bramley *et al.*, 2005).

Oestradiol plays a role in the regulation of the function of CL in many species. In female sheep, for example, exogenous oestradiol can either extend the life of CL or promote luteolysis, depending on the time of administration (Glass *et al.*, 1984). In ovine CL, the concentrations of cytosolic oestradiol receptors was estimated to be 6.9 to 19.0 fmol/mg protein, and these receptors also have a role outside the oestrous cycle. Furthermore, cytosolic receptors in the anterior pituitary and the uterus are lowest during the luteal phase, at the time progesterone levels in the blood are high (Glass *et al.*, 1984). In sheep oestradiol increases endometrial progesterone receptors *in vitro* which means that the decline in progesterone would allow synthesis of oestradiol receptors and subsequently oxytocin receptor synthesis earlier in animals having a short rather than a normal cycle (Khan, 2000).

There are many factors that can cause inadequate luteal function, such as the poor response to the LH surge after an injection of GnRH, which explains the deficiency in the final maturation stage of the follicle. It can also be caused by a short period of exposure of the developing follicles to secreted LH (Hunter *et al.*, 1986). The cytosolic oestradiol receptors in the ovine CL were found to be at their highest concentration at the end of the luteal phase of the oestrous cycle and the concentration of oestrogen was observed to be higher during the luteal phase than in the early or midluteal phase (Zieba *et al.*, 2000). The end of the luteal phase, a decrease in androgens and progesterone was observed and a significant increase in oestradiol secretion which reflects the increased FSH secretion in the blood, as FSH is one of the main factors which stimulate follicular growth and maturation. In addition, it is well documented that steroid hormones may play a role in regulating luteal function and they might also stimulate growth of a new generation of ovarian follicles (Zieba *et al.*, 2000).

Oxytocin and the endometrium have a regulating role in the function of the uterus. The concentration of oxytocin receptors in the endometrium of the ewe ovary changes during the oestrous cycle. During the middle of the luteal phase, oxytocin receptor density is increased in the endometrium as well as in the myometrium (Okano *et al.*, 1996). Furthermore, oxytocin stimulates $\text{PGF}_{\alpha 2}$ secretion via activation of calcium-dependent C kinase and phosphoinositol hydrolysis (Hu *et al.*, 2001, Okano *et al.*, 1996). Moreover, $\text{PGF}_{\alpha 2}$ and PGE_2 are secreted periodically by endometrial cells into the blood, resulting in pulse increase in its concentration (Krzymowski and Stefanczyk-Krzymowska, 2008).

1.3.6.2 Luteolysis of corpus luteum

Luteolysis is defined as the lysis or structural demise of CL. During normal luteolysis, two closely related events occur. Firstly, there is loss of function, i.e. capacity to synthesize and secrete progesterone (McGuire *et al.*, 1994b), followed by a loss of cells that comprise the CL (Knickerbocker *et al.*, 1988). In most animals luteolysis is dependent on the presence of a uterus, as a hysterectomy prevents or delays luteolysis, as shown in heifers (Anderson *et al.*, 1961, Malven and Hansel, 1964, Wiltbank and Casida, 1956), ewes (Wiltbank and Casida, 1956), and pigs (Anderson, 1966). Prostaglandin $\text{F}_{\alpha 2}$ is the factor in the uterus that initiates luteolysis (McCracken *et al.*, 1970) in most non-primate species. After production $\text{PGF}_{2\alpha}$ enters into the ovarian artery from the utero-ovarian vein via a counter current transfer mechanism (Ginther, 1974).

It is proposed that oestradiol from the growing follicle initiates the release of hypothalamic oxytocin (McCracken *et al.*, 1996), which in turn stimulates the release of a small quantity of uterine $\text{PGF}_{2\alpha}$ (Fairclough *et al.*, 1980). $\text{PGF}_{2\alpha}$ then initiates a positive-feedback loop which

involves the release of additional oxytocin and $\text{PGF}_{2\alpha}$ of both luteal (Tsai and Wiltbank, 1997) and uterine origin (Silvia *et al.*, 1991). Oxytocin, stimulates the synthesis and secretion of $\text{PGF}_{2\alpha}$ from the uterus in ewes (Walker *et al.*, 1997), heifers (Lafrance and Goff, 1985) and mares (Goff *et al.*, 1987). It has been proposed that the release of luteal $\text{PGF}_{2\alpha}$ amplifies the luteolytic signal in an autocrine and paracrine manner (Olofsson *et al.*, 1992, Tsai and Wiltbank, 1997, Tsai and Wiltbank, 1998).

Prostaglandin $\text{F}_{2\alpha}$ reduces blood flow to the corpus luteum and thus may cause luteolysis by depriving the gland of nutrients, substrates of steroidogenesis and luteotropic support (Pharriss *et al.*, 1970). Prostaglandin stimulates the endothelial cells of the corpus luteum to produce endothelin-1 (Girsh *et al.*, 1996, Ohtani *et al.*, 1998), a potent vasoconstrictive activator (Huggins *et al.*, 1993) and it also inhibits the steroidogenic activity of the luteal cells (Girsh *et al.*, 1996).

Prostaglandin acts by binding to specific receptors localized at large luteal cells (Helmer and Britt, 1987, Juengel *et al.*, 1996). These receptors belong to the seven-transmembrane family of G protein-coupled receptors (Abramovitz *et al.*, 1994, Graves *et al.*, 1995). Upon binding to high affinity receptors, $\text{PGF}_{2\alpha}$ induces the activation of membrane-bound PLC (Berridge and Irvine, 1984) via a stimulatory G protein (Miwa *et al.*, 1990). Phospholipase C catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate (IP3) (Davies, 1988b) and 1,2-diacylglycerol (DAG). Increased cytosolic concentrations of IP3 result in the release of free Ca^{++} from the smooth endoplasmic reticulum to the cytoplasmic compartment (Berridge and Irvine, 1984). Increased free Ca^{++} and DAG stimulate the catalytic activity of Ca^{++} -dependent protein kinase (PKC) (Nishizuka, 1986). This PKC is believed to

mediate many of the antisteroidogenic actions of $\text{PGF}_{2\alpha}$ in large luteal cells (McGuire *et al.*, 1994a, Wiltbank *et al.*, 1990, Wiltbank *et al.*, 1991). It is possible that apoptosis in large luteal cells is facilitated by PKC activation.

During luteolysis, T lymphocytes infiltrate the corpus luteum and secrete interferon- γ (IFN- γ), which stimulates the presentation of major histocompatibility complex antigens on the surface of the luteal cells (Fairchild and Pate, 1991). Interleukin-1 (IL-1), produced by macrophages, fibroblasts and endothelial cells (Paavola and Christensen, 1981) stimulate the production of $\text{PGF}_{2\alpha}$ in cultured bovine luteal cells (Nothnick and Pate, 1990). Production of bioactive TNF- α begins after the loss of the progesterone synthesis in the ovine corpus luteum (Ji *et al.*, 1991) but TNF- α could serve to complement the luteolytic activity of uterine $\text{PGF}_{2\alpha}$ during luteolysis by stimulating the synthesis of luteal $\text{PGF}_{2\alpha}$. In summary, immune cells and cytokines appear to play a role in luteolysis by regulating $\text{PGF}_{2\alpha}$ synthesis, steroidogenesis, and phagocytosis.

1.3.7 Controlling the oestrus cycle with CIDRs

Controlled internal drug release (CIDR) is a method of oestrus synchronisation in sheep. It has been used extensively as a method for the release of progesterone in sheep to mimic the normal luteal phase (Crosby *et al.*, 1991, Hamra *et al.*, 1989, Simonetti *et al.*, 2008, Wheaton *et al.*, 1993). Using a CIDR twice a day during a 12 day period resulted in a greater 3-day oestrus response in treated ewes compared non-treated. However, the data showed no differences in follicular development, conception rate, and prolificacy between treatment and control animals when the ewes received two CIDR's of progesterone (Dixon *et al.*, 2006). Other studies

resulted in a decrease in fertility in ewes treated with one CIDR of progesterone (Allison and Robinson, 1970, Johnson *et al.*, 1996, Robinson and Smith, 1967, Vinales *et al.*, 2001).

Ewes treated with progesterone and PGF_{α2} 24 hours before CIDR insertion or after removal, exhibited greater signs of oestrus than those which received PGF_{α2} only (Boland *et al.*, 1978, Dixon *et al.*, 2006). Using a dose of bST at day 5 before progestin removal increased the number of lambs born per ewe lambing with increased circulating concentrations of IGF-I during their pre-ovulatory period (Carrillo *et al.*, 2007). A large number of follicles were developed during the preovulatory period when ewes were treated for a long time with pFSH in conjunction with variable supplies of FSH and LH (D'Alessandro *et al.*, 2005).

Kohno, *et al.* (2005) used two methods for oestrous synchronisation, by controlled internal drug release (CIDR) and intravaginal cream containing 500 mg progesterone (P cream). They conclude that the P cream method was as effective as CIDR in inducing oestrus and ovulation and subsequent fertility in ewes during the non-breeding season. However, there was tendency for the mean P4 concentrations during the P cream insertion period to decrease early, and it is necessary to examine different sizes, densities, and materials for the sponges, used in the P cream method before future use.

1.3.8 Progesterone

Intravaginal progesterone sponges are commonly used to synchronise the oestrous cycle of ewes. The sponge mimics the action of corpus luteum through provision of an artificial source of progesterone sufficient to suppress gonadotrophin production. In addition removal of the sponge removes the progesterone block and induces synchronous re-instatement of

gonadotrophin release and subsequent ovulation in treated ewes (Hawken et al., 2005). In cattle the removal of the progesterone block at the end of the synchronisation protocol does not reliably result in a synchronous onset of ovulation due to varying stages of follicle development at sponge removal (Roche et al., 1999). Similar observation have been made in sheep synchronised during the breeding season, due to an impact of stage of the oestrous cycle at sponge insertion on follicle size at sponge withdrawal (Leyva et al., 1998).

In the ewe, each follicular wave consists of one to four ovulatory-sized follicles and the incessant follicular waves can be observed to emerge during the luteal and follicular phases of the oestrous cycle (Adams, 1999). Also in sheep, there have been no earlier attempts to ablate large antral follicles with the progestogen-oestradiol treatment prior to superovulation (Bartlewski et al., 2008).

1.3.9 Prostaglandin (PG)

This method is used to increase the overall efficiency of genetic improvement programmes. As PGF_{α2} is only effective in ewes with active corpora lutea, progestagen treatment for 10 to 14 days and an injection of gonadotrophin, usually eCG, and progestagen withdrawal has become the most widely used and versatile method for synchronizing oestrus in sheep (Cline et al., 2001). PG 600, containing 400 iu of eCG and 200 iu of hCG, is approved for inducing oestrus in gilts (Cline et al., 2001). PG 600 can be used instead of eCG to prepare ewes for natural breeding (Jabbar et al., 1994). The pregnancy rate was very poor in PG 600-treated ewes, but fertilization rate was approximately 75% (Maxwell et al., 1996). Moreover, oocyte quality per se would not seem to have been responsible for the poor pregnancy rate, although AI at an inappropriate time relative to ovulation might have been (Cline 2001).

1.3.10 Gonadotrophin Releasing Hormone GnRH

GnRH is one of the methods use to improve the reproductive performance of sheep by reduced pre-implantation losses (Khan et al., 2006). It has been shown that GnRH analogue on Day 12 post-mating can improve embryo survival resulting in higher pregnancy rates in cattle (Peters et al, 1992) and increased lambing rates and litter size in ewes (Beck et al., 1994). Beck et al. (1996) showed that treatment with GnRH resulted in higher plasma progesterone concentrations. This increased progesterone could enhance uterine function leading to increased conceptus development and production of the antiluteolytic embryonic protein (IFN- τ), which suppresses PGF $_{\alpha 2}$ release (Khan et al., 2006). In addition use of eCG and GnRH treatments have been shown to provide a more compact ovulation in ewes (Menchaca and Rubianes, 2004; Zeleke et al., 2005), thus providing the potential to increase the pregnancy rates following laparoscopic artificial insemination in sheep (Khan et al., 2006).

1.4 Differences in the oestrous cycles of ewes and ewe lambs

In sheep, the oestrous cycle is controlled by hormonal and physiological factors, as well as environmental factors. There is considerable variation in the oestrous cycle of ewe lambs, in particular at the beginning of the breeding season where silent heats are far more frequent in the ewe lambs than in adult ewes. Furthermore, these silent oestrous periods are likely to be a result of inadequate progesterone priming prior to first ovulation (Ward, 1980). Some studies conclude that the length of oestrus is shorter in lambs than in adult ewes, (Bathaei, 1996, Leftsson and Dyrmondsson, 1990) and that the onset of reproductive cycles start later in young female sheep than in adult sheep in the same season (Foster, 1981, Papachristoforou *et al.*, 2000).

The general oestrous cycle for adult ewes usually occurs at regular times compared to immature ewes. The oestrous cycle of the mature ewes is described in Section 2.4. Furthermore, ewe heat duration is, on average, 35 h in length (Hafez, 1952) and ovulation in ewes usually occurs towards the end of oestrus, approximately 24 h after the onset of the LH surge. Dyrmondsson (1983) implies that the majority of ewe lambs have a regular oestrous cycle, and that their fertilisation rates are the same as for adult ewes (Quirke *et al.*, 1983a). However, ovulation is lower (Dyrmondsson, 1973a) and embryo mortality is higher compared with mature ewes (Downing, 1980, Hamra and Bryant, 1979). Moreover, Davies and Beck (1993) found that the maximum peak of LH concentration was lower and that the duration of release tended to be shorter in ewe lambs compared to adult ewes. However, Quirke (1981) did not find any difference between ewe lambs and adult in the peak of LH or ovulation rate.

1.4.1 Differences in ovulation, fertilization, conception and lambing rate between ewe lambs and ewes

Lambs ovaries are smaller and less well developed and lambs have a lower ovulation rate than adult sheep (McKenzie and Terrill., 1937). The release of more than one ovum in ewe lambs is rare but it can occur in more prolific breeds, but despite this the ovulation rate is much lower than in mature ewes. Ovulation rates have been found to be different in Clun forst ewe lambs (1.07) and ewes (1.25) (Beck *et al.*, 1996b), but Walmsley *et al* (2004) found ova recovered from ewe lambs had significantly more abnormalities, although cleavage rates were similar. However, there is no evidence to suggest that ova from ewe lambs may be less developed than that ova from mature ewes (McMillan and McDonald, 1985).

In the majority of ewe lambs, fertilisation rates are similar to those observed in adult ewes, but the reproductive performance of ewe lambs is greatly reduced by excessively elevated rates of

embryo mortality (Quirke and Hanrahan, 1983) during the first stage of pregnancy (pre-implantation). Lane (1991) has reported that the sperm requires more than two hours to reach the oviducts of ewe lambs, which were mated at the first or third oestrus: this may partly explain the lower pregnancy rate of ewe lambs compared with adult ewes, along with the conception and lambing rates are lower in ewe lambs than in yearlings or adult ewes (Gordon, 1967). High ovum cleavage rates (80%) and low implantation rates (<40%) have been observed in Galway ewe lambs after various progestagen-PMSG treatments (Quirke and Hanrahan, 1977). It is possible that the eggs produced by ewe lambs do not have the same potential for development as those from older ewes, or that the conditions in the reproductive tract of the ewe lamb are less favourable for survival (Quirke and Hanrahan, 1977). The conception rate in sheep treated with progestagen was 70% to 80% (Gordon, 1997).

The lambing performance of ewe lambs, however, does not vary with breed along with management and environmental factors. Typically, 20 to 40% of animals that are mated during the breeding season remain barren (Dyrmundsson, 1983, Quirke, 1981a). Embryonic mortality in ewe lambs is commonly higher than in ewes and must therefore, contribute greatly to their relatively poor reproductive performance (Beck *et al.*, 1996b). Hare and Bryant (1985) reported that fertilisation improved on the second cycle ewe lambs, whilst Beck *et al.* (1996b) reported that fertilization rate and embryonic survival in ewe lambs can be improved by using oestrogen and progesterone to stimulate three oestrous cycles prior to mating.

1.4.2 Hormonal profiles of young and adult female sheep during the oestrous cycle and early pregnancy

With advancing pregnancy in sheep the pulstile secretion of LH decreases progressively (Al-Gubory *et al.*, 2003). An *in vitro* study of pituitary glands from pregnant ewes determined that

basal LH secretion falls from day 45 of pregnancy onwards (Fowler and McNeilly, 1997). The most prominent change in LH secretion was a decrease in concentration observed on and after day 20 of pregnancy. By day 60 of pregnancy there was a decrease in all the parameters of pulsatile LH secretion compared with those on day 10 of the oestrous cycle. Pulses of LH were either absent or infrequent at this time (Fowler and McNeilly, 1997).

Some work has been done with ewe lambs on the recognition of pregnancy and other maternal changes, and on hormonal profiles under different environmental conditions. Quirke and Gosling (1978) determined progesterone and LH concentrations in order to investigate the effect of nutrition on lambing rates in two breeds of ewe lambs, Fingalway and Galway. They found maximum progesterone levels of 1.5 ng/ml at Days 0-35 of pregnancy with slightly higher levels in Fingalway (prolific) than in the Galway (non-prolific) ewe lambs. The levels recorded were similar to those for non-pregnant ewes during Day 10-12 of the oestrous cycle. It seems that progesterone concentrations in pregnant ewe lambs are lower and more variable than those in adult ewes, with a transient fall in concentrations between day 20 and 30 post-mating (Downing, 1980).

Furthermore, Downing (1980) recorded that plasma progesterone concentrations in Clun Forest ewe lambs during early pregnancy was 1-3 ng/ml, whereas the comparable levels in mature ewes of the same breed were significantly higher (3.6-5.4) during the same period of pregnancy. The progesterone concentrations recorded in pregnant ewe lambs were similar to the non-pregnant ewes during day 10-12 of the oestrous cycle. Moreover, progesterone concentrations in pregnant ewe lambs showed a transient decrease between Day 20-30 of pregnancy. Quirke and Gosling (1981b) reported that there are different progesterone levels on

Day 18 of pregnancy (ewe lambs, 3.3 ng/ml; ewes, 5.3 ng/ml) but by Day 35 that the difference had decreased (ewe lambs, 3.1; ewes, 3.9 ng/ml). This decline in concentration around Day 18 mirrors the transient fall in progesterone reported by Downing (1980) and may be a contributory factor in embryo mortality.

Higher levels of growth hormone have been reported in ewe lambs as compared with ewes (ewe lambs, 5.0; ewes, 0.5 ng/ml) (Johnsson *et al.*, 1985). Prolactin concentrations were measured and found to be 75-125 ng/ml in 18-week old ewe lambs (Johnsson *et al.*, 1985). Davies and Beck (1993) compared the plasma hormone profiles in ewe lambs and ewes during the oestrous cycle and early pregnancy and reported that no difference in mean LH concentration, pulse rate or amplitude occurred on Day 13 in pregnant ewe lambs and ewes. However, LH concentrations were significantly greater in ewe lambs than in ewes on Day 28 of pregnancy but progesterone concentrations were lower. Nevertheless, no difference was found in mean prolactin concentrations during early pregnancy between ewe lambs and ewes.

There is a relationship between age and metabolic hormones and metabolites which influence ovulation rate and embryo survival in sheep. These may play an important function in controlling reproduction in juvenile ewes (O'Callaghan and Boland, 1999). Insulin may play a role in controlling the ovulation rate in sheep, which is recorded at a higher level in ewes than ewe lambs during oestrus; this might be caused by an increased rate of lipolysis and reduced glucose utilisation (Davies and Beck, 2003). Overall, the oestrous cycle of ewe lambs shows some deficiency in hormones and glucose release compared with adult ewes.

1.5 Ovarian response to hormonal stimulation during the oestrous cycle and early pregnancy in ewes and ewe lambs

During the oestrous cycle, the pulsatile release $\text{PGF}_{\alpha 2}$ is important for luteolysis, which is controlled by oxytocin (Wathes and Lamming, 1995). Oxytocin is secreted from the posterior pituitary gland (Walker *et al.*, 1997) and CL (Theodosis *et al.*, 1986). During luteolysis, secretion of $\text{PGF}_{\alpha 2}$ depends on the binding of oxytocin to endometrial receptors (Flint and Sheldrick, 1983). At the end of the luteal phase, the luteolytic signal is activated in response to an increase in the number of oxytocin receptors (Sheldrick and Flint, 1985). In the ewes and ewe lambs treated with buserelin, an increase in progesterone concentrations was observed and consequently a decrease in oestradiol concentration (Beck *et al.*, 1996a). It is possible that the increase in progesterone concentration and decrease in oestradiol levels could suppress endometrial oxytocin receptor formation, which leads to a reduction of $\text{PGF}_{\alpha 2}$ secretion and thereby embryo survival (Khan *et al.*, 2006). Treatment with GnRH demonstrated that the luteolytic signal was stronger in ewe lambs compared with ewes and this may be one of the major causes for high embryo loss in ewe lambs. There is also evidence to suggest that treatment with GnRH failed to improve embryo survival in young female sheep. Abnormal ovarian steroid pattern during the luteal phase could also change the level of uterine oxytocin receptors (Beck *et al.*, 1994b). Treatment with buserelin induced additional corpora lutea in ewes but not in ewe lambs. There are higher levels of progesterone in ewes than in ewe lambs, which may be more environmentally favourable to growth and development of the embryo during the early stages of gestation in ewes treated with buserelin (Khan *et al.*, 1999), but not in ewe lambs (Beck *et al.*, 1994b, Khan *et al.*, 1999). However, Beck *et al.*, (1996a) reported that there was no long term effect on overall mean progesterone concentrations in ewes treated with buserelin, even though concentrations tended to be higher between 2 to 6 h post-treatment.

Moreover, results showed that GnRH treatment during the mid-luteal phase caused a transient increase in circulating progesterone concentrations (Foster *et al.*, 1980).

The fertility and conceptus mass of ewe lambs did not increase with GnRH treatment. Even though GnRH can stimulate conceptus growth, there is no evidence to suggest that the ovine conceptus has binding sites for GnRH (Beck *et al.*, 1994b, Khan *et al.*, 2007). The increase in progesterone levels after being treated with GnRH on day 12 of pregnancy suggests that GnRH, through LH release, may provide luteotrophic stimulation to the corpus luteum. This stimulation may be in the form of conversion of small luteal cells to large luteal cells and consequently, secretion of higher concentrations of progesterone (Farin *et al.*, 1988)

In addition, neither fertility nor conceptus mass improved when ewe lambs were treated with hCG, but there was an increase in the number of placentomes after hCG treatment. This may occur due to promoting implantation, which may help the improvement of long term embryo growth and survival. Although, hCG treatment significantly increased the number of placentomes in ewe lambs, the number of placentomes was lower than that in ewes. Treatment with hCG increased plasma progesterone concentration (Khan *et al.*, 2007).

Khan *et al.* (2007) reported that sheep following hCG on day 12 of the oestrous cycle showed a higher progesterone concentration in the blood plasma of ewes than in ewe lambs. Nephew *et al* (1994) reported that in ewes treated with hCG on day 11.5 of estrous cycle there was an increase in plasma progesterone concentrations. The increase in oestradiol concentration after this treatment may play a role in the establishment of pregnancy, as there is evidence to show

that in the ovine blastocyst, the synthesis of oestradiol coincides with blastocyst elongation (Khan *et al.*, 2007).

The increase in progesterone levels after being treated with hCG on day 12 of pregnancy suggest that hCG, through its LH-like action LH, may provide luteotrophic stimulation to the corpus luteum. That stimulation may be in the form of the conversion of small luteal cells to large luteal cells, which consequently secrete higher concentrations of progesterone (Farin *et al.*, 1988). hCG is reported to have a half life greater one day, compared to LH, which has a half life of few hours in heifers (Schmitt *et al.*, 1996). Higher plasma concentrations of progesterone were observed in the hCG group compared with the GnRH group suggesting that the mechanism by which GnRH and hCG stimulate luteal function could be similar (Khan *et al.*, 2007). There was an increase in LH concentration in ewes and ewe lambs as a response to GnRH treatment, which may be responsible for the decrease in the conversion of stage 2 luteal cells into stage 3 luteal cells (Schmitt *et al.*, 1996a).

In GnRH treated sheep, there was no difference in LH basal release between ewes and ewe lambs, but there was a higher peak and a longer duration of LH release in adult ewes compared with juvenile ewes. When animals were treated with GnRH, no difference was observed in basal plasma FSH between ewes and ewe lambs in the control group, but there was a greater increase in FSH concentrations in young ewes as compared to adults after treatment with GnRH (Davies and Beck, 1993). Moreover, there is a greater increase in FSH in plasma of the ewe lambs compared with that in ewes due to GnRH treatment response, which suggests that pituitary gonadotrophins might be regulated by negative feedback of oestradiol, and inhibin a

lesser extent may well be due to an absence of large follicles on day 12 in ewe lambs (Khan *et al.*, 2007).

1.5.1 The administration of exogenous hormone to improve reproduction performance in sheep

Some studies were conducted to improve the reproductive performance ewe lambs using exogenous hormone supplementation. Pregnancy rate was increased in Clun Forest ewe lambs when treated with 500 iu PMSG (Davies, 1988b) which is similar to the findings of Quirke (1979b). In addition, using 500 iu of PMSG reduced embryonic mortality by 8.5%, but using 1000 iu PMSG increased the incidence of embryonic mortality by 2% and an approximate decrease of 20% in the fertilization rates of PMSG treated ewes. PMSG reduced embryo mortality by 18.9% and 6.1% respectively for lambs treated with 500 and 1000 iu of PMSG. The ovulation rates increased from 1.15 to 2.0 to 2.71 respectively with control, 500 iu and 1000 iu PMSG respectively (Davies, 1988b).

An experiment was conducted using post-mating progesterone supplements by subcutaneous injection or intra-vaginal passaries but both failed to improve either ewe lamb lambing percentage or the number of ewe lambs subsequently maintaining their pregnancies. Interestingly, progesterone concentrations during the luteal phase tended to be higher prior to mating in subsequently pregnant ewe lambs than in barren ewes. However, it was concluded that the treatment of ewe lambs with supplementary progesterone for the first 30 days of pregnancy was unsuccessful in decreasing embryonic mortality rates (Davies, 1988b).

In ewes treated with 375 mg or 250 mg of progesterone from day 7 until day 14 after mating, pregnancy rate was increased by 23% and litter size by 40% in yearlings and 23% in mature

ewes. Pregnancy rate and litter size were increased with an increased dosage of progesterone. The results showed the plasma levels of progesterone concentration in ewe plasma treated with 375 mg was higher compared with those treated with 250 mg of progesterone (Theodosiadou *et al.*, 2004). Plasma progesterone concentration of ewes increased after an injection of GnRH on day 12 post-mating (Khan *et al.*, 2007) or on day 11.5 by hCG (Nephew *et al.*, 1994) and, therefore, an improved implantation and embryo survival in ewes but not in ewe lambs. The GnRH acts via the release of LH, while hCG has the same function as LH, may offer luteotrophic stimulation to CL. Furthermore, ewes treated with GnRH or hCG slightly improved the ovarian function of ewes but not of ewe lambs (Khan *et al.*, 2007). This could be because ewe lambs CL did not respond to these hormones, or due to a stronger luteolytic response in ewe lambs than in ewes (Beck *et al.*, 1994b, Khan *et al.*, 2007, Khan *et al.*, 2006). Treatment with GnRH did not affect CL diameter (Reyna *et al.*, 2007).

1.5.2 Maternal recognition of pregnancy

In vertebrates, a large family of autocrine and paracrine modulators of fibroblast growth factors (FGFs) were found (Ornitz and Itoh 2001). The first FGF identified, basic FGF or FGF2, initially was described as a mitogen for mice (Armelin 1973). The action of FGFs are not restricted to cell growth, but also influence several cellular events, including cell migration, cell differentiation, cell survival, angiogenesis and tumorigenesis (Powers *et al.*, 2000 and Bottcher and Niehrs, 2005). Many FGFs function as key regulators of mammalian development (Bottcher and Niehrs 2005). FGF2 is implicated in regulating embryonic and early placental development in various species. Immunoreactive FGF2 protein is detected in the uterine lumen of cyclic and pregnant cows at day 17 after oestrus (Michael *et al.*, 2006). Uterine production of FGF2 prior to placental attachment, has been investigated in the ewe, and was found to be

produced in the endometrium and placenta during mid-and late gestation and is implicated in regulating angiogenesis (Reynolds and Redmer 2001).

Implantation of the conceptus is a critical developmental step in mammalian reproduction and involves a complex interaction between embryonic trophoctodermal cells and receptive maternal endometrium (Martin et al, 2004). During implantation, the uterus undergoes remodelling to create an optimized environment for embryonic and fetal growth. The generation of this remodelled receptive endometrium is dictated by many factors including peptides, steroid hormones, cytokines and growth factors, which are sequentially secreted by both embryonic and maternal tissue (Martin et al, 2004). A major messenger in the communication that occurs between the conceptus and the endometrium of sheep is the type I interferon- τ (IFN- τ), which is anti-luteolytic and transiently secreted by the extra-embryonic trophoctoderm during the peri-implantation period (day 12-21 of pregnancy) (Martal et al, 1979).

1.6 Ovarian function in vitro

Numbers of studies were conducted to study ovine (Bister *et al.*, 1999, Tambe and Nandedkar, 1993) and bovine (Driancourt, 2001, Kruip and Dieleman, 1989) follicles *in vitro*. Where steroid hormones measured after follicles cultured in different media and for different time of incubation. As the method of culture and the time of oestrous cycle influence both the amount of steroids secreted by follicles in culture (Kruip and Dieleman, 1989). In sheep, steroidogenesis follicles is dependent on size of the follicle, degree of atresia and stage of oestrous cycle when the follicle is dissected from the ovary and culture media (Moor *et al.*, 1973).

1.6.1 In vitro studies of follicular function

At the onset of the breeding season in sheep, *in vitro* steroidogenesis was identical, in terms of basal activity, to the measurement at the middle of the breeding season, but the follicular response was largely enhanced. Moreover, follicles obtained at the end of the breeding season featured higher progesterone and lower oestrogen production and were not influenced by LH or FSH. The basal progesterone secretion was comparable to that observed in the middle of the breeding season (Bister *et al.*, 1999). Steroidogenesis could amend oocyte function, as the ability to develop to the blastocyst stage after *in vitro* maturation and fertilisation appears to be related positively to oestradiol concentrations in follicular fluid (Moor and Trounson, 1977) and related negatively to androgen concentration in follicular fluid (Andriesz and Trounson, 1995). Oestradiol production *in vitro* was affected by age, as Driancourt *et al.* (2001a) reported; the oestradiol output of calf follicles was low because their aromatase activity was reduced markedly and fifteen and three spots differed qualitatively or quantitatively when the two-dimensional patterns of follicular proteins of calves and cows were counted. Moreover, the pattern visualized in cow and calf follicular fluid after western blotting with an antibody increased compared with α inhibin.

The steroidogenic function of bovine follicles influenced *in vitro* by some factors, such as the culture method, culture time and gasphase and essential factors such as follicle size, stage of cycle and quality (Kruip and Dieleman, 1989).

A study conducted by Mann (1992) found that the rate of oestradiol secretion measured *in vivo* was some 20 to 30 fold higher than that seen after 2 h incubation, which indicated that the incubation procedure was giving a reasonable indication of the pattern of the secretory capacity of individual follicles. Furthermore, a lower secretion rate during the incubation period would

be expected as a result of the lack of gonadotrophic stimulation and the absence of capillary blood flow. Moreover, there is a narrow correlation between size and quality *in vivo*, and a relative efficiency of steroid production *in vitro* was found by Moor *et al.* (1978). The small and medium follicles seem to be fully capable of producing high levels of oestrogen, but this capacity seems to be inhibited *in vivo*. Oxygen seems to stimulate the metabolic activity of the granulosa cells including the hydroxylation reaction that forms an essential part of the aromatizing system (Kruip and Dieleman, 1989). *In vivo* maturation, increase in follicle size results in a increase in oestrogen and decrease in testosterone in the follicular fluid (Kruip and Dieleman, 1989) and *in vitro*, large follicles (oestrogenic follicles) produced a larger amount of oestradiol during culture than small follicles. While large non-oestrogenic follicles were found to have a lower concentration of inhibin in the follicular fluid releasing less inhibin during culture than large oestrogenic follicles, the magnitude of these differences was much lower than was the case for oestradiol (2 fold for inhibin compared with 10-20 fold for oestradiol) the large oestrogenic follicles contributing 90% of the total oestradiol production (Mann *et al.*, 1992, Webb *et al.*, 1989). Moreover, more oestrogenic follicles were found in ewes in the early and mid-follicular phase than in the luteal phase or late follicular phase. Oestradiol is the main hormone secreted by the large oestrogenic pre-ovulatory follicles. However, a great amount of inhibin was secreted from non-oestrogenic atretic follicles as well as small follicles (Mann *et al.*, 1992). *In vitro* oestradiol production was influenced by number of granulosa cells, and this led to the variation in *in vitro* oestradiol production. Moreover, the breed affected oestradiol production. Is also affected by breed that there is no breed difference exists between Romanov and Ile-de-France ewes in the number of granulosa cells in oestrogenic follicles. On a per ewe basis, that Merino x Scottish Blackface ewes had significantly fewer granulosa cells in

oestrogenic follicles compared to Finnish Landrace ewes with the Finnish Landrace x Scottish Blackface ewes intermediate number (Webb *et al.*, 1989).

Moor and Crosby (1987) reported that granulosa cells do not remain stable when cultured *in vitro* but have changes influenced by gonadotrophin hormones similar to that occurring *in vivo*. Consequently, steroid secretion will increase and changes in the morphology of the cell will occur. As a response to this change the pattern of progesterone secretion would change when granulosa cells are grown in a monolayer culture. The capability of granulosa to influence the quality of the maturation of oocytes when cultured at the same time and in a similar condition, suggests that follicle cells have a powerful function in supporting the development of the oocyte. The communication between the granulosa cells, cumulus and oocyte occur via a gap junction (Moor and Crosby, 1987) where many metabolites pass through.

Zieba *et al.* (2000) isolated granulosa cells on day 9 and 12 of the ovine oestrous cycle and found that cells secreted a great amount of progesterone and androgen with a tiny amount of oestradiol. This finding was confirmed by steroid hormones found in extracts of CL harvested at the same period.

There are some peptides present in the ovarian follicular fluid that govern follicular growth and development (Monniaux *et al.*, 1997, Webb *et al.*, 1999). These include inhibin, IGF-I, TGF- β and EGF which control follicular division (Armstrong and Webb, 1997, Campbell and Scaramuzzi, 1995, Monniaux *et al.*, 1997). Follicular fluid consists of a numbers of nutrits, such as hormones, growth factors, enzymes and electrolytes. The function of follicular fluid is to support the oocyte in undergoing meiosis and to protect the oocyte from proteolytic attack as

well as to enhance sperm attraction (Avery *et al.*, 2003), and the composition of follicular fluid changed via the oestrous cycle (Orsi *et al.*, 2005). Moreover, plasminogen activator and inhibin are macromolecular components of follicular fluid (Yanagishhita and Hascall, 1979). The source of these components is the blood, in addition to other components (synthesised products of follicular cells and oocytes). Oestradiol, epidermal growth factor, fibroblast growth factor, meiosis activating sterols, inhibin, and actvin play a major role in regulating the oocyte and follicular growth the secretion (Nandi *et al.*, 2008).

1.6.2 Studies on luteal function in vitro

Progesterone secretion by ovine luteal tissue *in vitro* has used quite different tissue preparations and incubation conditions (Bramley *et al.*, 2005). Moreover, progesterone secretion has been studied for enzymically dispersed luteal cells (Braden *et al.*, 1989), used luteal slices (Hunter *et al.*, 1988) and luteal minces (McNeilly *et al.*, 1992). Moreover, the incubation media used, the amount of tissue or number of cells per incubation, and the duration of incubation used (from 3 to 12 h) varied markedly between studies. Bramley *et al.* (2005), results differed from those of previous studies in a number of important respects. Firstly, he used hCG in preference to oLH whereas Hunter *et al* (1986, 1988) have shown that ovine luteal tissue binds hCG with an affinity 3-30-fold higher than that of oLH. Secondly, Bramley *et al.* (2005) used luteal minces as the conditions used by others to disperse luteal tissue significantly reduced luteal LH receptor levels, may possibly compromising steroidogenic response *in vitro* and disrupted cell-cell communication may be important for normal progesterone secretion (Grazul-Bilska *et al.*, 2001a). Under the conditions of incubation used in Bramley *et al.* (2005) progesterone secretion increased linearly with the duration of incubation in treated and untreated tissue for up to 3 h. However, basal progesterone secretion increased progressively

with increasing duration of incubation, leading to an apparent flattening of the dose-response. These factors make it difficult to directly compare data on steroid secretion and hormonal responsiveness with data obtained by other groups, but Bramley *et al.*, (2005) suggested that this was the reason for the failure of some studies (Braden *et al.*, 1989, Chemineau *et al.*, 1993) to demonstrate a significant progesterone response to LH *in vitro*.

The basal secretion of progesterone *in vitro* by CL from day 4 of the cycle was significantly greater than for day 12 of the cycle, CL when measured both at mid-breeding season and in anoestrus ewes. However, increasing total LH receptor levels coupled with reduced LH secretion in response to feedback from rising luteal progesterone levels between day 4 and 12 would also contribute to a decrease in receptor occupancy (Bramley *et al.*, 2004). Furthermore, during the mid or late breeding season, luteal sensitivity to hCG *in vitro* was significantly depressed on day 4, although the difference in sensitivity to hCG was not significant on day 12 because of the large between-animal variability. This variability may reflect the difference in responsiveness between ewes in which CL is beginning to fail and ewes with a CL that continues to function up to day 12. Alternatively, the defect present on day 4 may be corrected in a proportion of animals as the CL matures (Bramley *et al.*, 2004). CL induced in anoestrus Scottish Blackface ewes that treated that were pretreated with progesterone and given GnRH injections of increasing frequency had a similar lifespan on ewes that ovulated spontaneously in the mid-breeding season, secreted similar levels of progesterone *in vivo*, and showed a similar response to oLH injection *in vivo*. Moreover, although these CLs tended to be smaller than CL formed during the mid-or late-breeding season, and had a significantly lower luteal progesterone content (Bramley *et al.*, 2004). Data from Bramley *et al* (2005) indicate that luteal function in Scottish Blackface ewes was severely compromised in CL-induced

pharmacologically during anoestrus by the –P/GnRH bolus (but not the + P/GnRH ramp-treatment) regimen. However, luteal function in Scottish Blackface ewes was also subtly compromised in spontaneously ovulating ewes as they approached the spring breeding season-anoestrus transition. These changes in luteal function at different stages of the season did not appear to be due to inadequate levels of LH receptor occupancy.

LH has a significant effect on progesterone secretion in the earlier rather than the final stages of luteal development, whereas dbcAMP increases progesterone production across all days of the oestrous cycle. The conclusion of this study has shown that progesterone production by luteal cells depends on the stage of luteal development, LH and dbcAMP effects (Borowczyk *et al.*, 2007). Bovine luteal cells without contact secrete less basal progesterone and LH-induced progesterone than those with established cellular contacts. Cellular contacts were required for maximal stimulation of progesterone synthesis by LH (Del Vecchio *et al.*, 1996).

1.6.3 Oocyte maturation

Mammalian oocytes experience extensive molecular reprogramming during the maturation period. During maturation some stored mRNA are mobilized and translated to provide the protein required for the completion of meiosis and for the acquisition of developmental competence. At the same time, other mRNAs and proteins required by oocytes during the period of prophase arrest are degraded (Moor *et al.*, 1998). Furthermore, the signal of these molecular changes in the oocyte originate from the follicle cells as a response to precisely timed changes in circulating levels of gonadotrophins in the 48 h before ovulation. It may, therefore, contribute to alterations in these physiological signals which would, in turn, alter the sequence of molecular events in the oocyte itself (Moor, 1998). Progression of nuclear events in the

oocyte maturation is also controlled by cytoplasmic components (Eppig, 1994). Structural rearrangement of organelles and changes in protein synthesis take place during cytoplasmic maturation (Motilk J, 1990).

Factors such as animal age and breed as well as the stage of the oestrous cycle, body condition, diet and ovarian morphology have an effect on oocyte maturation as well as growth and development (Gordon, 2003). In mammalian ovaries oocytes are arrested in the initial stage of meiotic division until the release of the pre-ovulatory gonadotrophin that stimulates the resumption of meiosis (Richard and Sirard, 1996). Immature oocytes develop from germinal vesicle (meta phase I) to metaphase II in the final stage of maturation. During this stage meiosis is again arrested for the second time and is normally completed upon fertilization (Lonergan, 2000). Oocyte maturation consists of cytoplasmic and nuclear maturation, which means that the oocyte has the ability to progress during the meiosis stage.

The key to the ability of the oocyte to achieve successful development is oocyte competence. Three steps are required for oocyte competence.

(a) Ability to resume meiosis. For example, in the cow, the oocytes acquire the capability to form a metaphase plate when reaching its full size just before antrum construction. In some farm animals the capacity to attain metaphase I is acquired before the capacity to reach metaphase II (Hampl and Eppig, 1995).

(b) Ability to cleave following fertilization. This step normally happens without stimulation and in large animals an occur in fully grown oocytes in the absence of fertilization. If cleavage does not happen, it is not clear whether this is due to a dysfunctional sperm that is unsuccessful in

activating the oocyte or to the oocyte not having the ability to undergo the first cell division (Gomez *et al.*, 1998).

(c) Capability to develop to the blastocyst stage. In standard culture conditions cattle oocytes should reach the blastocyst phase within 6-9 days to have a successful chance of inducing a pregnancy (Barnes and First, 1991).

Low blastocyst rates with oocytes obtained from pre-pubertal sheep have also been attributed to a high incidence of polyspermy resulting from delayed and erratic cortical granule migration (O'Brien *et al.*, 1996). Embryo mortality rate is high during the first stage of pregnancy in sheep (Grealley *et al.*, 1996), which has a negative effect on the reproductive performance of these animals.

1.6.3.1 Oocyte maturation in vitro

The number of high quality oocytes collected from each ovary is important to investigate *in vitro* maturation and produce embryos in cattle (Leibo and Loskutoff, 1993). Several methods are used to obtain oocytes from slaughtered sheep ovaries; follicular dissection, and the aspiration of oocytes, puncture and slicing. The slicing method produces more debris, which interferes in the search for oocytes (Wani *et al.*, 2000). Slicing is the cheapest and most abundant source of oocytes, and is more commonly used for ovine oocyte recovery (Agrawal, 1995) than the aspiration of follicles (Slavik *et al.*, 1992, Watson *et al.*, 1994).

In vitro matured oocytes have a significantly lower capacity for future development than those produced *in vivo* (Dieleman *et al.*, 2002, Rizos *et al.*, 2002). The most commonly used culture medium system consists of hormones, serum or albumin (Bavister *et al.*, 1992, Thompson,

2000, Younis *et al.*, 1989). FSH, LH and steroids play an important function in controlling oocyte maturation (Moor and Trouson, 1997, Younis *et al.*, 1989, Zuelke and Brackett, 1990). Accardo *et al.* (2004) reported that without gonadotrophins in the oocyte culture medium there was no expansion of the granulosa cells. At the same time, using FSH and LH were shown to improve the expansion of granulosa cells in different mammalian species (Choi *et al.*, 2001, Singh, 1993, Zuelke and Brackett, 1990). In cattle, using FSH with the oocyte maturation medium increased the rate of the oocytes expansion of granulosa cells after *in vitro* maturation, however, there were no significant effects on nuclear maturation rates observed (Choi *et al.*, 2001).

Different types of culture media are used for oocyte maturation. Serum and serum albumin (BSA) are commonly used as a protein source in mammalian oocytes culture media (Ali and Sirard, 2002, Keskinetepe and Brackett, 1996, Wang *et al.*, 1997). Normal birth weights were observed following the transfer of embryos cultured in media containing BSA and amino acids in place of sera (O'Brien *et al.*, 1997). In cows, using oestrogens in the oocytes culture media has a negative effect, in both denuded and oocytes with cumulus, in the nuclear maturation, including abnormal dispersion of chromosomes and on subsequent embryo development (Beker *et al.*, 2002). However, the main role of oestrogens in follicular and oocyte maturation, ovulation and embryo development depends on animal species (Moudgal *et al.*, 1996). The composition of culture media might influence the different stages of oocyte maturation. Currently the tissue culture media (TCM 199) is widely in use as a basic *in vitro* maturation media for mammalian tissue culture. Fetal calf serum (FCS) is one of the culture media commonly used as supplements for *in vitro* oocyte production (Gordon, 2003) along with polyvinyl alcohol (PVA).

In sheep, the heterogeneity in diameter of the oocytes isolated from antral follicles of 4-6 mm was much higher than those observed in other species. Moreover, in many species, the ability of oocyte to resume and complete meiosis *in vitro* has been attributed to the oocyte diameter (Ledda *et al.*, 1999b-b, Otoi *et al.*, 2000). Shirazi and Sadeghi (2007a) reported that in sheep, there is a correlation between follicular size and oocyte diameter, because oocyte diameter influences the meiotic progression. However, no relation was found between oocyte diameter and the ability to reach to MII.

Oocyte maturation is one of the most important stages for the *in vitro* production of embryos (Mariana Groke Marques, 2007). Research in this area has shown that mammalian *in vitro* produced embryos have to some extent a lower developmental competence and quality compared with their *in vivo*-derived counterparts (Knijn *et al.*, 2003, Warzych *et al.*, 2007). Van de Leemput *et al.* (1999) reported that the *in vitro* development potential of *in vivo* matured oocytes is double that of corresponding *in vitro* matured gametes.

1.6.3.2 In vitro studies of oocytes from ewes and ewe lambs

Oocyte competence indicates the ability of an oocyte to undergo fertilisation and develop to an embryo. *In vitro* methods have long been used for embryo production (IVEP) to enhance genetic gain and shorten the generation interval in order to accelerate the improvement of productivity performance in sheep. Female age is an important factor affecting oocyte competence and therefore *in vitro* embryo production. thus using young females as sources of oocytes is important to produce embryos, which accelerate improving ewe lambs reproductive function (Armstrong, 2001).

The TCM medium is the most common used culture medium, supplemented with hormones, FCS or serum albumin. Also the *in vitro* technique allows the use of a large number of oocytes, which is beneficial for genetic improvement and to accelerate research (Thompson, 2000). Media components are established to help the metabolic and nutritional requirements of cultured oocytes. Furthermore, FSH and LH play a crucial role in the regulation of oocyte maturation (Moor and Trouson, 1997). Bovine serum albumen is one of the additives to the maturation medium which minimizes variability in the maturation medium. The components of the culture media and conditions of culture can influence the meiotic regulation (Accardo *et al.*, 2004).

Some studies concluded that a lower oocyte developmental competence was present in juvenile ewes compared with mature ewes (Ledda *et al.*, 1997, O'Brien *et al.*, 1996, Revel, 1995) whereas others reported that the capacities for young sheep paralleled those of mature sheep (Armstrong *et al.*, 1997a, Irvine *et al.*, 1993). Furthermore, some of these studies indicated that adult sheep have a greater capacity for *in vitro* development and that there was more polyspermic fertilization and a reduction in the *in vitro* development capacity with young sheep oocytes (O'Brien *et al.*, 1996). The mean pregnancy and survival rate is significantly higher for adult oocyte donors than for pre-pubertal donors (Gonzalez De Bulnes *et al.*, 2003), although Kochhar *et al.* (2002a) reported that the time-dependent change in cytoskeletal and chromosomal status of lamb-derived oocytes were noticeably similar to those of adult ewe-derived oocytes. In addition, oocytes derived from juvenile post-pubertal ewe antral follicles can be matured and fertilised *in vitro* with good results (Armstrong *et al.*, 1992) although the development capability of pre-pubertal ewes was less than mature ewes, resulting in a lower number of viable embryos (Palma, 1993). It has been suggested that this was caused by a defect in cytoplasmatic maturation (Duby *et al.*, 1996). Levesque and Sirard (1994) reported that there were some differences in protein pattern and content of oocytes

derived from ewe lambs in comparison to oocytes derived from ewes. However, the rates of *in vitro* maturation, fertilization and cleavage were similar for both juvenile and adult sheep oocytes. Nevertheless, close observation revealed that ewe lamb oocyte fertilization is associated with an increased polyspermic incidence. This may be because the gonadotrophic hormone receptors are inadequate to enhance the oocyte developmental capacity at this age (O'Brien *et al.*, 1996). Blastocysts derived from the oocytes of young ewes had a visible inner cell mass and did not differ morphologically from those derived from adult ewe. However, delayed embryonic development of ewe lamb-derived embryos compared with adult-derived embryos was observed. The majority of blastocysts from adult ewe-derived oocytes developed on day 6 of culture compared to blastocysts from pre-pubertal-derived oocytes which usually developed one or two days later (Grazyna Ptak, 1999, Presicce *et al.*, 1997).

The use of pre-pubertal animals as an oocyte donor would reduce the generation interval and initiate earlier progeny investigation within some breeding programs. In addition the oocytes are released from their inhibitory influences of their follicles and cultured *in vitro* with a competent condition are able to spontaneously resume meiosis and complete maturation. Important physiological processes normally occur during oocyte growth, in both the nucleus and the cytoplasm allowing the oocyte to achieve final development competence. However, the oocytes harvested from follicles less than 2mm in diameter did not resume meiosis (Ledda *et al.*, 1999b-a). In addition, Moor and Trouson (1997) indicated that in the oocytes recovered from large follicles, meiotic development were higher than those derived from small follicles, as there was a positive correlation between follicular size and meiotic development when follicles cultured *in vitro* (Cognie *et al.*, 1998).

In mammalian animals the resumption of meiosis by the oocyte requires endocrine and paracrine signals as well as the presence of vital molecules for receptor mediated and intercellular signal transduction. The series of processes consequently include germinal vesicle breakdown (GVBD), chromosome condensation and progression to metaphase of meiosis 1 (MI), extrusion of the first polar body and arrest at MII of second meiosis (Kochhar *et al.*, 2002b). Oocyte maturation is also controlled by cytoplasmic components (Eppig *et al.*, 1994) and the structural rearrangements of organelles and changes in protein synthesis take place during cytoplasmic maturation (Motilk J, 1990). Moor and Crosby (1986) had reported that synthesis of new proteins are required for meiotic resumption, which are necessary for GVBD in fully developed sheep oocytes.

Oocytes derived from juvenile ewes are different to those derived from adult ewes in some *in vitro* parameters tested but were similar in the other aspects. The time dependent changes in the cytoskeletal and chromosomal status of lamb-derived oocytes were unusually similar to those of mature ewe derived oocytes. Kochhar *et al.* (2002b) also reported that the development potential was significantly ($P < 0.05$) in oocyte from adult ewes than those from ewe lambs. The majority of oocytes derived from matured ewes passed through meiosis I at 12 hour, anaphase-telophase at 18-20 hour and reached MII stage at 24 hour of *in vitro* maturation. Ewe lambs oocytes patterns were similar although the proportion of oocytes that reached MII was higher at 26 hour (78.6%) than at 24 hour of maturation (60%) (Kochhar *et al.*, 2002b), whereas Ledda *et al.* (1997) and O'Brien *et al.* (1996) reported that MII occur at 24 hours.

Some studies indicated that ovaries from pre-pubertal sheep can be stimulated to grow with exogenous hormones. Moreover, the *in vivo* viability of *in vitro* produced embryos as assessed at lambing, following embryo transfer was similar for blastocysts derived from mature ewes and pre-

pubertal ewes treated with progestagen/FSH (Earl, 1994, O'Brien *et al.*, 1997, O'Brien *et al.*, 1996). This is contrary to the study conducted by Ledda *et al.* (1996) who found that oocytes obtained from untreated pre-pubertal ewes demonstrated a lower developmental potential than those obtained from adults. *In vitro* maturation technique remains inadequate to complete oocyte growth (Ledda *et al.*, 1999a).

1.7 Embryo development in sheep

After the oocyte is penetrated by the sperm (fertilisation), a new embryo is formed which takes place in the ampulla. The fertilisation process activates the resumption of the second meiotic division with the extrusion of the second polar body and configuration of the pronuclei. The male and female pronuclei migrate to the centre of the cell, the membranes of the pronuclei then break-down and release the chromosomes. The oocytes then experience mitotic division, known as cleavage (Hafez, 1993). In general, the cleavage of mammalian eggs is very slow compared with the other species in the animal kingdom (12-24 hours separately). The first cleavage occurs within 24 hours after fertilisation (Oppenheimer, 1984). This cleavage is a normal meridional division. During the second division one of the two blastomeres divides meridionally while the other divides equatorially (Gulyas, 1975). In mammals, blastomeres do not become separated at the same time and embryo development does not increase regularly. The changes from maternal to zygotic control occur at the 2 cell stage in mice and goats (Prather *et al.*, 1989).

Embryo cells start to divide and produce a morula and this stage of development consists of 8 to 16 blastomeres (Oppenheimer, 1984). In sheep, the morula includes small groups of internal cells surrounded by larger group of peripheral cells. However, a large section of the external cells become trophoblast cells, which develop into chorion known as a part of placenta in order to

secrete hormones during the foetal stage which is ended by the delivery (Gilbert, 1997). Later the trophoblast cells secrete fluid into the morula to form a blastocoele, the morula does not have an internal cavity as in the early stage. In the trophoblast cell the inner cell mass is displaced to one side, this form is called a blastocyst. Until this time the embryo is still surrounded by the zona pellucida. Towards the end of this stage the blastocyst contains roughly 300 cells, and then starts hatching. On day 9 approximately 90% of the ova have hatched (Oppenheimer, 1984).

The epithelial cells in the uterine wall develop cytoplasmic protrusions, whereas the trophoblast cells develop several microvilli. These changes happen in order to prepare the uterus and the foetus for implantation at day 15 of pregnancy and at that time the embryo may reach up to 22 cm in length (Hunter, 1980). It's governed by numerous growth factors which classified families such as:

- a) Epidermal growth factor family of Fibroblast growth factor (EGF), including neuregulin (EGF) and transforming growth factor- α (TGF- α).
- b) The family of insulin-like growth factors (IGFs) includes (IGF- I & II).
- c) Fibroblast growth factor family (FGF) comprising nine types of (FGF).
 - d) Platelet-derived growth factor (PDGF).
 - e) Transforming growth factors- β (TGF- β).
 - f) Cytokines and consist of leukaemia inhibitory factor (LIF), colony stimulating factor-1 (CSF-1), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins and interferons (Nicola, 1994).

1.8 Protein expression and identification in sheep ovarian tissue

Proteomics and genomics are information sciences involving the generation of very large data sets, which need to be organised, stored and made accessible in logical ways. They both have requirements in terms of low-level and high-level analysis. Proteomics relies heavily on protein sequence databases, which in turn are usually generated from the translation of nucleic acid sequences. Whilst nucleic acid sequence databases are growing exponentially, the challenge for proteomics is that most of the information in sequence databases is for a small number of, mostly, model organisms. Proteomics is an emerging area of research that directly evaluates protein expression by resolving, identifying, quantifying, and characterizing proteins utilizing a variety of techniques. These include high resolution two-dimensional polyacrylamid gel electrophoresis, tandem mass spectrometry, and computer analysis (Celis, 1998). Protein separation technology has existed for 40 years based on electrophoresis and polyacrylamide gels (Corthals, 2000). Protein spots, which appeared to be surface labeled, were cored from the two-dimensional gels and submitted for Tandem mass spectrometry analysis (Coonrod *et al.*, 2002).

The high rate of embryo mortality is one of the major problems facing the mammalian breeding industry because it affects reproductive efficiency, genetic improvement and the development of embryo-related biotechnology. To address the problem of embryo mortality *in vitro* fertilization is vital in order to obtain an improved understanding of normal embryo development. Moreover, the knowledge of the protein content of embryos from fertilisation to the development of the blastocyst is important (Grealy *et al.*, 1996), such as protein synthesis (Frei *et al.*, 1989).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used to separate and characterize several proteins from sheep follicles (Driancourt *et al.*, 1996), rams' semen (Cardozo *et al.*, 2008) and cattle oocytes (Ali and Sirard, 2002). High-throughput proteomics has been established using a combination of high-resolution 2D-PAGE, highly sensitive biological mass spectrometry and the rapidly growing protein and DNA databases (Gevaert and Vandekerckhove, 2000).

Treatment with exogenous hormones and studies of luteal function *in vivo* suggest that luteal and follicular tissues are less steroidogenic in ewe lambs than in ewes using hormone measurement (Beck *et al.*, 1996, Khan *et al.*, 2006 & 2007). Although it is unclear if other factors may have influenced the observed plasma hormone concentrations. For example, the metabolic rate is faster in ewe lambs than in ewes and consequently the hormone clearance would be faster in blood of ewe lambs than in blood of ewes.

Furthermore, the lower ovulation rate observed in ewe lambs may be associated with fewer oestrogenic follicles and corpora lutea. The objective of this study was to compare ovarian function in ewes and ewe lambs using *in vitro* studies. Moreover, this study was performed on tissue (follicles, oocytes and corpora lutea) from both age groups of animal during the same time of the oestrous cycle. This study was designed to use different media and different incubation times for hormones estimation, protein synthesis and oocytes maturation. All the experiments were conducted in similar conditions for both ewes and ewe lambs tissue to minimise the factors affecting animal performance described above. The comparison was made between ewes and ewe lambs without external effects, which may affect the accuracy of the results and any differences attributed to the different age of the animals.

Chapter Two

General Materials and Methods

2.1 Introduction

This chapter contains an explanation of the materials and methods that are common to most of the experiments, with the specific materials and methods described for each experiment. The chemicals and reagents were bought from Bio-Rad, Sigma or Fisher Scientific. For personal protection and to avoid sample and equipment contamination, gloves were worn at all times and changed frequently. Forceps, scalpel blade handles, scissors and pipette tips were autoclaved at 121°C. Radioimmunoassay was done in designated areas, which were cleaned each time after use.

Distilled water (dw) and double distilled water (ddw) was used throughout the experimental work, using Direct-QT™ Millipore (High Wycombe, Buckinghamshire, HP14 4JH, U.K.), water purification system. Tissue cultures were carried out in a SANYO CO₂ Incubator (Model MCO- 17AIC, Sanyo Electric CO.Ltd, Japan) at 38°C and 5% CO₂ atmosphere. All sterile solutions were filtered using Acrodisc® Syringe 0.45µm HT Tuffryn® Membrane Low Protein Binding Non-Pyrogenic REF. 4184 (Gelman Laboratory).

2.2 Animals

The experimental work was conducted at Peithyll Field Station, Capel Dewi, which is situated 8 km east of Aberystwyth at an altitude of 70-90 m, and at Morfa Mawr Field Station, which is located 18 km south of Aberystwyth altitude. The annual rainfall for this area is approximately 1000 mm, with some yearly variation and both locations have a mild coastal climate. The ewes used in the experiments were Walsh Mountine, Speckle and Scotch black face, and approximately 5 years of old; The ewe lambs were 7-8 months old Texel x Mule. The

experimental work was carried out during the breeding season. Animal information is shown in appendices, 2A, 2B and 2C.

2.3 Management of animals

All the animals were maintained at pasture. The ewes were feed with 500 g of concentrate (sugar beet pellets) per day and the lambs with 250g of concentrate per day. Hay or silage was available and the water was supplied *ad libitum* during scarcity of green grass. Each animal was tagged with a numbered tag and some animals were re-tagged with a large plastic tag to allow easy identification of procedural animals at a distance. Raddled teaser rams were introduced to the groups from time to time for oestrus detection. Animals were divided into groups, depending on the stage of the oestrous cycle, and slaughtered between day 9 to day 12 post oestrus.

2.4 Collection of ovaries and preparation of tissue

The ovaries were collected within approximately 30 minutes of slaughter at the local abattoir, washed once with warm phosphate buffer saline (PBS) and placed into a pre-warmed flask containing 10 % of PBS and antibiotics (10 ml Penicillin- Streptomycin solution sterile filtered endotoxin tested from Sigma Aldrich) at 37°C and brought back to the laboratory within one hour of collection time. Some ovaries from both ewes and lambs were placed in dry ice immediately after collection for oocyte electrophoresis.

.

2.4.1 Collection of follicles

Follicles were dissected out from ovaries, using sterilized forceps and scalpel blade. Atretic follicles were avoided and healthy follicles were recognized by the presence of capillaries and clear fluid. Each follicle was cleaned of connective tissue and adipose tissue during the

separation process; the follicles were placed in a covered clean petri dish with TCM199 with 10% antibiotics in a water bath at 33°C. Using a sterile micrometer gauge, the follicles were classified according to size: small ($\leq 2\text{mm}$), medium (3-4mm) and large ($\geq 5\text{mm}$). The follicles were cultured as described for the individual experiments. Media were collected and stored at -20°C for hormone assay and the follicular shells were stored at -80°C, prior to estimation of protein concentrations and electrophoresis. During the processing of follicles in the laboratory the work was carried out in a clean laminar flow cabinet which was washed every time before use with 70% ethanol using sterile tissue (autoclaved before used). The water bath was warmed up for 30 mins before using each time. In addition, TCM199 medium was always placed in the autoclave 24 h before use.

Both follicular fluid and media were diluted into three different dilutions to determine the exact dilution for hormones determination (1:10; 1:100; 1:1000). However, the most accurate dilution (1:100) was used.

2.4.2 Collection of CL

CLs were dissected out from ovaries following the same method described in Section 2.4.1. Using sterile forceps and scalpel blade, CLs were cleaned of tissue and carefully washed three times in pre-warmed TCM199 with 10% of antibiotics. After this they were cut into equal pieces according to the purpose of the experiment (as described later in each experiment). Pieces were weighed (mg) and gently minced using a scalpel blade before culture. Tissue and media samples were diluted for progesterone estimation with three types of dilution used as described in section 2.4.1. However, the best dilution was found to be 1:10 for both CL tissue and media.

2.5 Hormone radioimmunoassay

2.5.1 Progesterone

Follicular fluid, CL tissue, the media from culture follicles and CL and plasma progesterone concentrations were analysed by RIA as described by Corrie, Hunter and Macpherson (1981) and modified by Law, Baxter, Louge, O'Shea and Webb (1992) for a direct assay procedure. The tracer used was progesterone 11 α glucuronide (125 I) iodotyramine (Amersham International Plc, Amersham, UK), diluted with assay buffer to give 12000 cpm in 100 μ l and then 1 mg/ml ANS (8-anilino-1-naphtalenesulphonic acid; Sigma Chemical Co., Poole, UK) was added to the tracer to dissociate steroids from plasma binding proteins. The standard (Sigma, P-0130) was diluted to give 7 standard curve points starting from 0.125-10 ng/ml whereas progesterone antiserum were supplied by the Antibody Production Unit Roslin, Scotland, and was used in a dilution of 1: 49. The second antibody (Sac-cel AA-SAC1, IDS Ltd., Tyne and Wear, UK) was raised in rabbits and used as supplied. Low, medium and high progesterone concentration samples were used for quality controls in each assay.

The assay buffer consisted of 9.51g NaH₂PO₄ 2H₂O (sodium dihydrogen orthophosphate; Fisher Scientific), 4.79g Na₂HPO₄ (disodium hydrogen orthophosphate; Fisher Scientific), 1.0 g NaN₃ (sodium azide; BDH, Poole, UK), 9.0 g NaCl (Fisher Scientific) and 1.0 g gelatine (BDH Chemical Ltd., Poole, UK). All buffer salts were dissolved in 800 ml of distilled water. The gelatine was dissolved in 200 ml of distilled water by gentle warming and finally both these solutions were mixed together slowly. The pH was then adjusted to pH 7.4.

On day 1, triplicate sets of standards (100 µl) and duplicate aliquots of unknowns (100 µl) were made up to 150 µl with assay buffer. Non-specific binding tubes, in triplicate, comprised 250 µl of assay buffer. Then 100 µl of first antibody (1:20000) was added to all the tubes (except for the total counts and non-specific binding tubes) and 100 µl of tracer (10-12000 cpm) was added to all the tubes, which were then mixed and incubated overnight at 4°C.

On day 2, 100 µl of the second antibody (Sac-cel) was added to all the tubes (except total counts), these tubes were then mixed and incubated at room temperature for 30 min. One ml of distilled water was added to all tubes (except total counts) and they were centrifuged at 3000 rpm for 5 min in a pre-cooled centrifuge. The supernatant was aspirated and the pellet counted for 1 min on a gamma counter (1270 Gamma Master: LKB Wallac, Stockholm).

2.5.2 Oestradiol-17β assay

Follicular fluid and follicle media oestradiol-17β concentrations were determined by RIA using a diagnostic kit (E₂ MAIA: Serono Diagnostic Surrey, UK) with the adaptations described by Mann *et al.* (1995). The lyophilised ¹²⁵I-labelled oestradiol was dissolved in assay buffer so as to give 10,000 cpm in 50 µl of buffer. The first antibody was diluted in assay buffer at a ratio of 1:7. The second antibody was used as provided in the kit. The standard (provided in the kit) was diluted to give 9 standard curve points ranging from 0.062-16 pg/50µl.

The assay buffer consisted of 9.66 g NaH₂PO₄·2H₂O (sodium dihydrogen orthophosphate; Fisher Scientific), 5.3 g Na₂HPO₄ (disodium hydrogen orthophosphate; Fisher Scientific), 2.0 g NaN₃ (sodium azide; BDH, Poole, UK), 9.0 g NaCl (Fisher Scientific), 1.0 g gelatine (BDH,

Poole, UK) and 3.72 g diaminoethanetetra-acetic acid (EDTA; Fisher Scientific). All buffer salts were dissolved in 750 ml of distilled water whereas gelatine was dissolved in 200 ml of distilled water by gentle warming and finally both these solutions were mixed together slowly by continuous stirring and made up to 1 litre. The pH was then adjusted to 7.6 and the buffer was stored at 4°C.

2.5.2.1 Assay procedure

Triplicate sets of standards (50 µl) and duplicate aliquots of unknowns were made up to 250 µl with assay buffer. Non-specific binding tubes, in triplicate, comprised 300 µl of assay buffer whereas total bound tubes (n = 3) contained 250 µl assay buffer. Then, 50 µl of first antibody (1:7) was added to all the tubes (except total counts and non-specific binding) and 50 µl of tracer (¹²⁵I-labelled oestradiol) was added to all tubes. Total count tubes (n = 3) received ¹²⁵I-labelled oestradiol alone. All these tubes were mixed and incubated for 2 h at room temperature. Free and antibody-bound tracer were separated by incubating with 100 µl of the second antibody (provided with the kit) for 10 min, followed by the addition of 1 ml of phosphate buffer saline (PBS). All the tubes (except total count tubes) were centrifuged at 6°C for 10 min at 3000 rpm and aspirated. Tubes were counted for 1 min on a gamma counter (1270 Gamma Master: LKB Wallac, Stockholm). Moreover, all the data hormones assays were transferred to logarithm transformation in order to have them within normal distribution.

2.6 Sample preparation for electrophoresis

Four different types of tissue were used for protein analysis; follicular shells, follicular fluid, oocytes and luteal tissue. Two different treatments were used for sample preparation, and two types of protein precipitation were used according to tissue type in order to get rid of

contaminating species such as nucleic acids, salts, polysaccharides and lipids, to ensure clear spots on the gel. These treatments are described according to the sample tissue.

2.6.1 Protein purification (precipitation)

Two methods of protein purification were used as explained in the following description:

2.6.1.1 TriChloro Acetic acid CHCL3O 2 (TCA) in acetone.

This method is widely used for protein precipitation (Jefferies et al, 2000). Luteal tissue, follicular shells and follicular fluid samples were extracted using this method. Twenty grams of TCA (Sigma) was dissolved in 70 ml acetone which was made up to 100 ml with acetone, mixed well and placed on the bench at room temperature.

One ml aliquots of defrosted samples were added to 1ml of ice cold 20 % TCA in acetone, vortexed and then placed for one hour in a freezer (-20°C), followed by 15 minutes of centrifugation at 13000xg and 4°C. The supernatant was removed and discarded. The pellets were then washed with 1 ml of 100 % acetone vortexed and placed in the freezer for 30 minutes, followed by centrifugation for 15 minutes as described above. This was repeated twice. The supernatant was then removed and discarded. After the second wash the samples were placed in the freezer with the cover open to allow the acetone to evaporate, and the pellets to dry for approximately 15-20 minutes. White pellets were formed at the bottom of the tubes. The samples were sonicated twice for 2 seconds each to release the pellets; the samples were now ready for protein estimation. All the preparation was done in an ice box.

2.6.1.2 Perfect FOCUS™ Protein Purification kit.

TCA extraction might improve sample purification but it is not as ideal as it should be; therefore the Perfect Focus Kit Method (Perfect-FOCUS™ for preparing Low Conductivity Samples for IEF/2D- Gel Electrophoresis. St. Louis, MO USA) was used to obtain good follicular shells, follicular fluid and luteal tissue samples for gel electrophoresis.

Before performing the Perfect Focus Kit Method, the protein concentration in the samples was measured. In an ice box, 300µl UPPA-I was added to 100 µg of protein sample in a 1.5ml micro centrifuge tube which was mixed well and incubated for 15 minutes at 4°C. Three hundred micro litres of UPPA-II was then added to each tube, which was then vortexed and centrifuged for 5 minutes at 13,000 xg and 4°C. The supernatant was then removed and discarded. The tube was centrifuged for a further 30 seconds, the supernatant again being removed and 40 µl of FOCUS-Wash was added on top of the pellet followed by 5 minutes of centrifugation. The supernatant was again removed and discarded. Twenty five micro litres of ddw was added to the tube, which was then vortexed. One ml of OrgoSol buffer, pre-chilled at -20°C with 5 µl of SEED, was added and then the tube again vortexed to suspend the pellet, after which it was incubated for 30 minutes at -20°C. The tube was vortexed for 30 seconds and centrifuged for 5 minutes at 13,000 xg and 4°C to form a tight pellet. The supernatant was removed and discarded and the samples placed in the ice box with the cover open for air drying. Three to five hundred µl of buffer C was added to each sample. The samples were now ready for protein estimation.

2.6.2 Protein estimation

It is important to estimate the concentration of protein in each sample before it is used and then calculate the amount of protein to be loaded per gel. The protein concentration in the sample was measured using Sigma-Aldrich Bradford reagent method (Bradford, 1976) according to the

manufacturer's instructions (Sigma). Bovine serum albumen (BSA; Sigma Aldrich) was used as a standard curve. Stock concentrations of 20µg/ml, 15µg/ml, 10µg/ml, 5µg/ml, and 2.5µg/ml BSA were made and used to construct a protein standard. The absorbance at 595 nm (A_{595}) was measured. Protein concentration was then estimated on each sample.

2.7 Electrophoresis

2.7.1 One dimensional SDS - PAGE gel

2.7.1.1 Mini sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE),

Mini gels were run using 7cm gels using the mini Protean[®] II system (Bio-Rad).

2.7.1.1.1 12% separating 7cm gels (12%T, 3.3%C):

The glass plates were cleaned with warm soapy water and allowed to air dry before they were ready to use as a casting gel using Bio-Rad Mini Protean[®] II. The amounts of reagents used are shown in the table below:

Notes

Separating gel 12 %	7 cm	
Acrylamide 30%	4.21 ml	30% acrylamide bis- acrylamide solution, Bio-Rad
SDS buffer PH 8.8	2.5 ml	PH 8.8 (Sigma)
ddw	3.34 ml	Double distilled water
APS 10 %	37.5 µl	Ammonium per sulphate (0.1 g in 1 ml ddw) Sigma
TEMED	10 µl	N,N,N',N'-Tetra-methyl-ethylenediamine (Bio-Rad)

The above ingredients were slowly mixed, using a 1000µl pipette. The solution was poured against the largest glass plate until the marker on the plate was reached. The upper surface of the gel was then covered with 2-propanol (for molecular biology: Sigma) and the gels were allowed to polymerise (about 30-45min) at room temperature; then the 2-propanol was removed, the gels rinsed twice with dw and dried using a filter

2.7.1.1.2 4% Stacking gel:

Notes

Stacking gel	7 cm	
Acrylamide 30%	375 μ l	30% acrylamide bis- acrylamide solution, Bio-Rad
Stacking gel buffer	625 μ l	PH 6.8
ddw	1.5 ml	Double distilled water
APS 10 %	12.5 μ l	
Temed	3.75 μ l	Tetramethylethylenediamine (Bio-Rad)

The above ingredients were mixed gently and poured on top of the separating gel. Plastic combs were then located in the stacking gel. For the large gels, 2 μ l of propanol was placed on top of the stacking gel and allowed to set at room temperature. When the stacking gel had completely solidified, the Bio-Rad Mini Protean[®] II was assembled in preparation for sample loading and gel running.

2.7.1.1.3. Running buffer: The 10 x Tris glycine electrophoresis buffer was diluted 1:10 and poured into the gel holding tank.

2.7.1.1.4 One dimension 7cm gels electrophoresis

Gels were made for ewe and ewe lamb oocyte samples, using the mini Protean[®] system (Bio-Rad). One part of the sample buffer (buffer C1) was mixed with 3 parts of sample (20 μ l per well), heated to 100 °C, for 5 mins, vortexed and centrifuged for 3 min at 12,000 xg rpm before loading on the gel. The first and the last wells of the gel were filled by 5 μ l of reference marker (pre-stained molecular weight, Sigma). A constant voltage of 70V was applied while the sample migrated through the stacking gel. The voltage was then increased to 80V through the separating gel until the electrophoresis dye front had reached the bottom of the gel (from 1-2 hrs).

2.7.1.2 Experion™ Pro260 (Experion system)

This technique was used to separate protein according to the molecular weight, using a small amount of sample (4µl). The Experion Pro260 reagents and materials were purchased from Bio-Rad.

2.7.1.2.1 Preparing the gel stain and the filtered gel

The Pro260 gel (G) and Pro260 gel stain (GS) cryovials were placed on the bench to allow them to equilibrate at room temperature for about 15 mins and then briefly vortexed and centrifuged for 3-5 seconds. Twenty µl of GS was added to the tube of 520 µl gel, which was then vortexed for 10 seconds and centrifuged for a few seconds. The mixture was transferred to a spin-filter tube and centrifuged for 5 mins at 10,000 xg. The GS was then ready to use, or stored in a fridge at 4 °C.

To prepare the filtered G, the content of 520 µl cryovial was pipetted into a tube of Pro260 gel and then filtered as described above. After 5 mins of centrifugation the filter was removed and discarded. The gel was stored in the fridge at 4 °C ready to use, according to the manufacturer's instructions.

2.7.1.2. 2 Preparing the sample buffer

The sample buffer supplied with the experion kit was placed at room temperature for equilibration and vortexed gently before use. One µl β- mercaptoethanol (BME) was added to 30µl sample buffer for each chip run and then vortexed.

2.7.1.2.3 Preparing the samples and the Pro260 ladder

The reference ladder was supplied with the Experion kit and after equilibration to room temperature, 2 µl of ladder was added to 4 µl of sample buffer in a 0.5 ml microcentrifuge tube and 4µl of sample was mixed with 2 µl of sample buffer in a 0.5 ml microcentrifuge tube. The

tubes were then vortexed and spun down using a microcentrifuge for few seconds. The samples and the ladder tubes were placed in a 95-100°C heating block for 5 mins and then cooled down and again spun for 15 seconds. Filtered ddw (84 µl) was added to each sample tube and vortexed briefly to mix before loading onto the chip.

2.7.1.2.4 Priming the chip

The chip was placed on the chip platform of the Experion station, 12 µl of filtered gel-stain solution was pipetted into the top right well (gel priming well) and the priming station was carefully closed. The pressure was then set to B and the time to 3 before beginning priming, which takes 60 seconds. The gel stain was removed and discarded and the chip was now ready for subsequent well loading.

2.7.1.2.5 Loading the samples and Pro260 ladder into the chip

There are four wells labelled GS 12µl of gel stain was loaded into each of the GS wells and one well was loaded with 12 µl of filtered gel (G). Six µl of each diluted sample was loaded into the sample wells, from 1-10, and 6 µl of diluted Pro260 ladder was pipetted into the ladder well (L).The chip was then ready to start the run. (All the reagents were prepared immediately before use every time).

2.7.1.2.6 Running the Pro260

The chip was placed on the platform of the Experion electrophoresis station before starting the run. When the process was completed, the chip was carefully removed from the platform and the electrophoresis station was cleaned with 800 µl ddw using the cleaning chip.

2.7.2 Two dimensional SDS - PAGE gels

2.7.2.1 First Dimensional SDS - PAGE gels:

Buffers used in sodium dodecyl sulphate polyacrylamide gel electrophoresis:

2.7.2.1.1 Separating gel buffer: 1.5 M Tris base (MW= 121.1); 45.41g., 10 ml of 10 % SDS(w/v) was dissolved in 200 ml of ddw using a magnetic stirrer; adjusted to pH 8.5 and then made up to 250 ml with ddw and stored in a fridge (4°C).

2.7.2.1.2 Stacking gel buffer: M Tris base (MW= 121.14). 6.05g; 0.4 % SDS 4ml (10% w/v) was made up to 100 ml with ddw and adjusted to pH 6.8.

Both the separating and stacking gel buffer gel buffers were left at room temperature for approximately 15 mins before the pH was again checked and then stored at 4°C.

2.7.2.1.3 Running buffer: five litres of 10x TGS Tris/Glycine/SDS, buffer (1x solution: 25 mM Tris; 192 mM glycine, and 0.1 % (w/v) SDS, pH 8.3, Bio-Rad), was diluted 1:10 in ddw before use.

2.7.2.1.4 C1 buffer (sample buffer): 6 M Urea. 3.6g (Fisher), 1.5 M Thiourea. 1.14g (Fisher), 3 % CHAPS 300 mg, 66 mM DTT. 100 mg, 0.5 % ampholytes pH range 3-10. 50µl, one tablet of protease inhibitors (EDTA, Penzberg Germany) and some bromophenol blue. The above ingredients were dissolved in 5 ml ddw, using a magnetic stirrer and then made up to 10 ml, the buffer filtered by 0.45µM filter paper and stored in the fridge as 1 ml aliquots until used.

2.7.2.1.5 Equilibration buffer: The equilibration buffer was made with 6.7 ml 1.5 M Tris -HCL (final concentration. 50 mM) pH 8.8., 72.0g urea (FW 60.06, Sigma), and final concentration. 6M., 69 ml glycerol final concentration 30 % (87 % v/v, Sigma), 4.0 g SDS (FW 288.38, Sigma) final concentration 2% w/v., ddw up to 200 ml with few grains of bromophenol blue

(Sigma). The ingredients were dissolved using a magnetic stirrer, and filtered by 0.45µM filter paper and stored in 12 ml aliquots at -20 °C.

2.7.2.1.6 Agarose buffer: One litre of 0.125 M Tris was made and adjusted to pH 6.8, stored at room temperature prior use. Half a gram of agarose (Agarose electrophoresis grade, Life Technologies, Paisley, Scotland) was dissolved in 100 ml of agarose buffer and heated using a microwave until completely dissolved, and then kept on the bench for use.

2.7.2.1.7 Isoelectric focusing of protein sample:

The protein sample was mixed with the sample buffer (buffer C1, section d). One hundred or 200 µg/ml of protein was used to rehydrate 17 cm (pH 3-10& 5-8); the linear or non linear immobilised pH gradient (IPG strips, Bio-Rad), 300 µl of solution (from the sample and sample buffer) was applied per strip. The first dimension was run using Protean[®] IEF (Bio-Rad). Protean[®] IEF cell set as shown in the table below:

	7 cm	11 cm	17 cm
Rehydration volume	135 µl	200 µl	300 µl
Start Voltage	0V	0V	0V
End Voltage	4000V	8000	10000
Volt-Hours	8- 10000V-hr	20- 35000 V-hr	40- 60000 V-hr
Ramp	Rapid	Rapid	Rapid
Temperature	20 °C	20 °C	20 °C

The strips were actively rehydrated overnight (from 16-20 hrs) prior to use. After protein focusing, the strips were moved into the equilibration buffer (section e). One hundred and twenty mg of DTT (dithiothreitol, Molecular Biology Grade Promega Corporation USA) were dissolved in 12 ml of equilibration buffer and 6 ml of this solution per strip was applied in a disposable

tray, which was placed on an orbital shaker for 15 mins. This was followed by the same amount of equilibration buffer with 250 mg of Iodoacetamide (IAA, Bio-Rad), again placed on the shaker for 15 minutes. The strips were then washed with dw and dried and were now ready to be loaded onto the gel for the second dimension.

2.7.2.2 Second dimension SDS-PAGE.gel running

The two gels were fixed on the cool core after the agarose had solidified and placed in a gel holding tank and filled with 1.5 litre of SDS running buffer 10 % (section c), where the inner space filled with 300ml of the same solution. To keep the gels running cool during the electrophoresis, cold water was run through the mid section of the gel tank. The power supply was set to give 200V per hour for the 17 cm gels and the running time was 7-8 hours. The end of the run was marked by the blue line reaching the end of the gel edge.

2.7.2.3 Gel casting for 2D gel electrophoresis: Before casting the gel one pair of glass plates were cleaned with hot water and soap, submerged overnight in 1% acetic acid, rinsed with ddw and allowed to air dry before being ready to use.

Casting of the separating gel:

Notes

Separating gel 12%	17 cm	
Acrylamide 30%	29.3 ml	30% acrylamide bis- acrylamid solution, Bio-Rad
Resolving gel buffer	17.5 ml	PH 8.5
ddw	23.2 ml	Double distilled water
APS 10 %	262 µl	Ammonium per sulphate (0.1 g in 1 ml ddw)
		Sigma
TEMED	70 µl	Tetramethylethylenediamine (Bio-Rad)

The above ingredients were well mixed and gently poured, layered with 2-propanol molecular biology, grade 99 %, Sigma was added on the gel surface, to remove bubbles and allowed to set before pouring the stacking gel. Residual 2-propanol was removed and the gel rinsed with ddw and then dried carefully using filter paper before pouring onto the stacking gel.

Casting of the Stacking gel: The solution was prepared as shown in the table below;

Notes

Stacking gel	17 cm	
Acrylamide 30%	1500 µl	30% acrylamide bis- acrylamide solution, Bio-Rad
Stacking gel buffer	2500 µl	PH 6.8
ddw	6000 µl	Double distilled water
APS 10 %	50 µl	
TEMED	15 µl	Tetramethylethylenediamine (Bio-Rad)

The gel components were mixed well and poured on top of the separating gel, layered with 2-propanol and allowed to set (like the separating gel). Residual 2-propanol was removed and then rinsed with ddw and dried using filters paper. Ready to load the IPG-Strip.

2.7.3 Protein gel staining

2.7.3.1 Silver staining

Gels were fixed in 50% (v/v) methanol (Fisher Scientific) and 5% (v/v) acetic acid (Fisher Scientific) and agitated gently for 40 minutes. After that the solution was completely removed and discarded using a fire hydrant vacuum system. The gels were subsequently washed in 50% (v/v) methanol for 20 minutes and washed in dw for a further 20 minutes. Then the gels were sensitised in 0.02 % (w/v) of sodium thiosulphate for 5 minutes followed by two rinses for 2 minutes in dw. The gels were submerged in chilled silver nitrate (Fisher) at 4°C, 0.1% (w/v) in a

shaker to stain them for 40 minutes, and then given two quick rinses in dw for 10 seconds. They were then moved to a developer solution, where 0.04% (v/v) of formalin in 2% (w/v) of sodium carbonate was used to develop the gels (the formalin was added just before use), for 10 to 15 minutes. The development was stopped, at the point when it appeared using acetic acid 1% (v/v) and the gels were then scanned.

2.7.3.2 Coomassie blue stain

Coomassie blue powder (2.5 g Fisher Scientific) was dissolved in 500 ml methanol and 100 ml of acetic acid and made up with ddw to 1000 ml and the solution was then filtered and kept on the bench. Gels were submerged in coomassie blue for one hour with gentle agitation. Excess stain was removed using a destaining solution, consisting of 400 ml methanol, 100 ml acetic acid and 500 ml dw, to destain the background gel. The solution was changed several times as it became saturated with stain. When sufficient spots appeared the de-staining solution was removed and the gel stored in 1% acetic acid until scanned.

2.7.4 Gel scanning and analyse

Gels were scanned after immediate development using a GS- 800 Calibrated Densitometer scanner; the images were captured and saved using PDQuest software before being exported to the TFT file. Progenesis (PG 200, PG 220 v 2006) software was used for image analysis.

Chapter Three

A comparison of basal steroidogenic function, *in vitro* and proteomics in follicles and corpora lutea from ewes and ewe lambs

3.1 Introduction

In ewe lambs, subfertility has long been documented as the main factor affecting productivity (Dyrmundsson, 1973a). Ewe lamb oestrous cycles and fertilization rates are similar to those in mature ewes (Dyrmundsson, 1983, Quirke *et al.*, 1983), whereas embryonic mortality rates are higher possibly due to deficiencies in hormone production (Davies, 1988, Davies and Beck, 1993). LH concentration is lower and its duration is shorter in the plasma of ewe lambs during both oestrus and early pregnancy. LH plays an essential function in the maintenance of luteal function. Furthermore, the lower concentration of LH could explain the decrease of progesterone concentration in ewe lambs plasma after day 13 post mating (Beck and Davies, 1994a, Davies and Beck, 1993, Khan *et al.*, 2007). This deficiency of progesterone may be due to a lower sensitivity of the lamb's corpus luteum to gonadotrophins. Furthermore, ewe lambs have a lower concentration of LH and prolactin secretion during oestrus, as well as lower LH and progesterone secretion during early pregnancy. These differences in hormone patterns may be correlated with subfertility in ewe lambs (Davies and Beck, 1993, Denamur, 1974).

Prolactin concentrations were also lower in ewe lambs than those in ewes during oestrus. These results may be attributed to lower sensitivity to oestradiol, reflect higher levels of dopaminergic inhibition, or be due to lower concentrations of oestradiol, all of which may contribute to the lower ovulation rate in juvenile ewes (Davies and Beck, 1993).

However, in the above studies hormones were directly compared and measured in the animal's plasma and considered despite variable factors, such as the number of oestrogenic follicles or corpora lutea, body size and difference in metabolic rates, as the clearance rates of hormones are potentially lower with increasing age. These factors may be responsible for the difference

in plasma progesterone and oestradiol concentration between ewes and ewe lambs, rather than the ability of ewe lambs corpora lutea and follicles to function normally.

Therefore an experiment was designed to investigate *in vitro* steroid hormone production from follicles and corpora lutea collected from ewes and ewe lambs between days 9-12 of the oestrous cycle. Samples were collected from ewes and ewe lambs of the same age and at the same stage of the oestrous cycle. All samples received identical treatment under the same conditions during the collection process and were cultured in identical media and under the same incubation conditions. Therefore, differences observed in this experiment could be attributed to age (ewes or ewe lambs) as all the other factors were controlled. In addition, experiments were conducted to determine the effect of media supplements and time on steroid secretion from follicles and CL. Furthermore, as differences in steroidogenesis between ewes and ewe lambs may be due to the absence of key enzymes or other proteins, corpora lutea and follicles were subjected to proteomics analysis.

3.2 Materials and methods

3.2.1 Experiment 1: Comparison of oestradiol and progesterone concentrations in media and follicular fluid of follicles from ewes and ewe lambs cultured *in vitro*

3.2.1.1 Follicles cultured for 24 h in TCM-199

Ewes, five years old mixed breed (n = 28) and Texel cross Mule ewe lambs (n = 36) at 7-8 months old were used in this experiment. All animals were slaughtered between day 9 to 12 of the oestrous cycle (oestrus = day 0). Ovaries were brought back to the laboratory within 1 to 3 hours of collection as described in section 2.4. Follicles were dissected out from the ovaries as described in section 2.4.1 using forceps and a scalpel blade. Follicles were collected in a clean sterile petri dish, which was in a pre warmed water bath at 33°C. The medium in the petri dish

was replaced every 30 minutes in order to keep follicles active during collection. Healthy small (2 mm), medium (3-4 mm) or large (≤ 5 mm) size follicles were then individually cultured in a covered sterile 24 well multi dish. Twelve randomly selected follicles from each size group were cultured in 2ml of pre-warmed TCM-199 plus 10% antibiotics and incubated as described in section 2.4. All follicles were cultured for 24 hrs. Oestradiol and progesterone concentrations were determined in culture media and follicular fluid after all samples were diluted 1:100 in both age groups. Hormone assays was done using RIA, as referred in section 2.5.

3.2.1.2 Follicular fluid from 24 h cultured follicles in TCM

After incubation, follicular fluid from ewes and ewe lambs follicles was collected as described in section 3.2.1.1. Follicles were gently dried on a filter paper using clean sterile forceps. Each follicle was carefully ruptured in a petri dish (5x1 cm) with 0.5 ml PBS using sterile forceps and scalpel blade and then the PBS with follicular fluid was collected in a small centrifuge tube (1.5 ml). All samples were stored at -20°C for hormone assays. Oestradiol and progesterone were determined as referred to in section 2.5.1 and 2.5.2.

3.2.1.3 Follicles cultured for 2, 4, 6 and 8 h in TCM

Ewes five years old, Welsh Mountain, Speckle and Blackface (n = 9) and Texel cross Mule ewe lambs (n = 18) at 7-8 months old, were used in this experiment. Medium size (32) and large sized (32) follicles were randomly selected from ewes and ewe lambs (64 follicles in total from each age group of animal). Follicles were individually placed in a 24 multiwell dish, cultured in 2 ml of TCM-199 with antibiotics and incubated at 37°C and 5% CO₂. Media from all follicles was collected and replaced at 2, 4, 6 and 8 h of incubation time. After incubation,

media from each follicle well was collected into 2 ml centrifuge tube and stored at -20°C until hormone assay. All follicles of the same size were collected in a sterilized petri dish and ruptured carefully in 0.5 ml of sterile PBS using forceps and scalpel blade. Follicular fluid collected in PBS and stored at -20°C until assayed. Before hormone assay, all samples were diluted 1:100 in oestradiol and progesterone assay buffer.

3.2.1.4 Follicles cultured for 24 h in TCM-199 with or without FCS

Ewes at five years old, Welsh Mountain and Black Face (n = 41) and ewe lambs (Texel cross Mule) at 7-8 months (n = 41) were used. The animals were managed as described in section 2.3. The animals were slaughtered between day 9-12 day of oestrus. The collection of ovaries and follicle classification were as referred in section 3.2.1 Whole follicles were individually cultured in a 24 well multi dish, with or without FCS and cultured similar to those described in section 4.2.1.1. Culture media was collected after 24 hours of incubation and stored at – 20°C until hormone assay as described in section 3. 2. 1. Oestradiol and progesterone levels were measured after all samples were diluted to 1:100 in assay buffer, the assays were done as referred in section 2.5.1 and 2.5.2.

3.2.2 Experiment 2

3.2.2.1 Comparison of progesterone secretion from CL cultured *in vitro* from ewes and ewe lambs

Ovaries were collected from ewes (n=41) and ewe lambs (n=41) at a local slaughterhouse and brought back to the laboratory as described in section 3.2.1. Forty one CL from each age group were dissected out from the ovaries and carefully cleaned of any surrounding tissue. The mean CL weights were similar between age groups of animal (ewes 702 mg, ewe lambs 713 mg, SED = 41.9).

Each CL was blotted dry on filter paper, weighed and cut into two halves. All halves were placed in a petri dish (100 x 20 mm) with 20 ml of Dulbecco's Modified Eagle's Medium (DMEM), and washed by moving them three times to the another dish containing fresh medium. Each half was then placed in an individual petri dish (60 x 15 mm), 6 ml of absolute ethanol (4°C) was then added to half the culture dishes, without incubation, to stop progesterone synthesis (control). Six ml of DMEM (100 ng/ml) was added to the other dishes and which were then incubated at 37°C, in an atmosphere of 5% CO₂. After 2 hours of incubation, CL halves and media were stored at -20°C until progesterone assay. Before hormone assay, all CL halves were thawed and each was gently homogenized in 5ml of sterile PBS with 10% antibiotics to prevent contamination and then diluted as described in section 2.4.2, for progesterone assay.

3.2.2.2 Comparison of progesterone secretion from CL cultured in different media

The same method described in section 2.4.2 was followed. CL from 98 ewes and 85 ewe lambs were collected after slaughter. The average body weights were 46.29 ± 0.88 kg and 35.25 ± 0.40 kg and condition scores were 2.39 ± 0.042 and 3.13 ± 0.03 for ewes and ewe lambs respectively. After CLs weighed. Twelve CL from each age of animals were randomly selected, the average weight was 792.67 ± 41.47 mg and 726.73 ± 28.45 mg for ewes and ewe lambs respectively. Each CL was divided into four equal pieces and then washed three times before incubation in pre-warmed TCM-199 before being cultured in (i) 2 ml of TCM-199 with 10% antibiotics (control), (ii) 2 ml TCM-199 plus 0.3 % bovine serum albumin (BSA), (iii) 2ml TCM-199 + 0.3 % Polyvinyl Alcohol (PVA) or (vi) 2 ml TCM -199 + 10 % fetal calf serum (FCS), in 24 well multi dish, at 37°C, in 5% CO₂.

After 24 h of incubation, media and luteal tissue were collected and separately stored at - 20°C for hormones assays. For hormone analysis, each CL was minced in 5 x 1cm petri dish using sterile forceps and a scalpel blade and put into a 2 ml centrifuge tube with 5 ml of PBS with antibiotics and gently homogenized. Before hormone analysis follicles and sample were diluted as described in section 2.4.2.

3.2.3 Proteomics of follicular shells and CL from ewes and ewe lambs

3.2.3.1 Follicular shells protein determination.

Ewes at five years old (Welsh Mountain, Black Face and Speckle, n = 28) and ewe lambs Texel cross Mule (n = 36) at 7-8 months old were used. All animals were slaughtered between day 9 to 12 of the oestrus (oestrus = day 0). Ovaries were brought back to the laboratory as described in section 2.4; Medium follicles (3-4 mm) were dissected out from each age groups of animal as described in section 2.4.1 and stored at -80°C without any treatment until used for proteomics.

3.2.3.1.1 Sample preparation

Follicle shells (n= 6) from each age were placed in 2 ml centrifuge tube with 1 ml of Tris 30mM (pH 6.5) with 10% of Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH, Roche Applied Science Nonnenwald 2 82372 Penzberg Germany) to inhibit protein proteases. Samples were then vortexed and homogenized before being centrifuged at 4°C and 100,000xg for 30 mins, to get rid of fat and debris. After centrifugation the supernatant was moved into a new centrifuge tube and the precipitate was removed and discarded. Protein purification was performed using the tri-chloro acetic acid in acetone method as described in section 2.6.1.1 and protein concentration was estimated by Bradford

method as described in section 2.6.2. The supernatant was then stored at -80°C until used for 2D gel electrophoresis. Samples preparation for 2DE was carried at an ice.

3.2.3.1.2 Two dimension electrophoresis (2-DE)

Using 17 cm pH 5-8 immobilised non-linear IPG strips (Bio-Rad), 300 µl of sample and sample buffer were applied per-strip. Protein samples of both age groups of animal were run at the same time on 2D electrophoresis gels as described in section 2.7.2. When the first dimension finished running, the strips were equilibrated as described in section 2.7.2.1.5. Three replicates gels from the same sample from each animal tissue were performed to create a average gel. After the second dimension was finished running, protein gels were stained using silver stain as described in section 2.7.3.1 and then all the gels were scanned as described in section 2.7.4 and saved on the lab computer until gel analysis. Gels analysis was performed using Progenesis software as described in section 2.7.4.

3.2.3.2 Corpus luteum

From section 3.2.1.1 the halves of CL which were kept at -80°C were used in this experiment, whereas the other halves had been cultured in TCM-199 with different concentrations of LH. Twelve halves of CL from each age group of animals were randomly selected, washed three times in TCM-199 and then dried on a filter paper before being used.

3.2.3.2.1 Sample preparation

In a clean petri dish, the luteal tissue were placed and completely minced using scalpel blade, before being carefully moved into a 50 ml plastic container with 10 ml of Tris 30mM at pH 6.5 with 10% anti proteases as referred in section 3.2.3.1.1. Samples were vortexed to mix the

tissue and the protein inhibitor and were then homogenised before being centrifuged at 4°C and 100,000xg for 30 mins. After centrifugation finished, the supernatant was moved into a new centrifuge tube and the precipitate removed and discarded. Protein purification was done by using the Trichloro acetic acid in acetone (TCA 20%) method as described in section 2.6.1.1 and then protein concentration was estimated by Bradford method as described in section 2.6.2. The supernatant was stored at -80°C in 1 ml aliquots. All of the above work was conducted in an ice box (4°C).

3.2.3.2.2 Two dimension electrophoresis (2-DE)

Protein samples in both age groups of animals were run at the same time on 2D electrophoresis gels as described in section 2.7.2, using 17 cm (3-10) immobilised pH non-linear IPG strips (Bio-Rad). 300 µl of sample plus sample buffer were loaded per-strip. When first dimension was finished, the strips were then equilibrated as described in section 2.7.2.1.5. Three replicates from the same sample of each animal tissue were used to create a average gel for each age group. Silver staining was used to stain the gels as described in section 2.7.3.1. All the gels were scanned as described in section 2.7.4 and saved on the lab computer until gel analysis. Gels analysis was done using Progenesis software as described in section 2.7.4.

3.2.4 Statistical analysis

All data was analyzed after log transformation in order to have all data in normal distribution. The data for oestradiol and progesterone in media and follicular fluid which was collected from tissue cultured for 24 h in TCM-199, was analyzed using ANOVA, in Genstat (8th Edition). Data collected from follicles at different times of incubation was analyzed by split-plot design and data for FCS section was analyzed using ANOVA.

Data from CL cultured in TCM-199 and cultured for 24 hours was analyzed using one-way ANOVA in GenStat (8th Edition). CL and the media from CL tissue cultured in four different media was analysed using two-way ANOVA was used (Animal ID as Blocking). 2 dimesion electrophoresis data were analyzed using biological value in progenesis software.

3.3 Results

3.3.1 Oestradiol and progesterone concentrations in the media from follicles cultured in vitro from mature ewes and ewe lambs

3.3.1.1 Oestradiol in media of follicles cultured in TCM-199

There was no significant ($P>0.05$) difference in oestradiol concentrations in the media after 24 h of incubation between the two age groups (Figure.3.1). Follicle size affected media oestradiol concentrations within age. Oestradiol concentration was significantly ($P<0.05$) higher in media from both ewe and ewe lamb follicles with increasing size (Figure.3.1). Oestradiol concentration in media from large follicles was greater than those of medium and small follicles, and, oestradiol concentration from the media of medium follicles was greater than those of the small follicles (Figure.3.1).

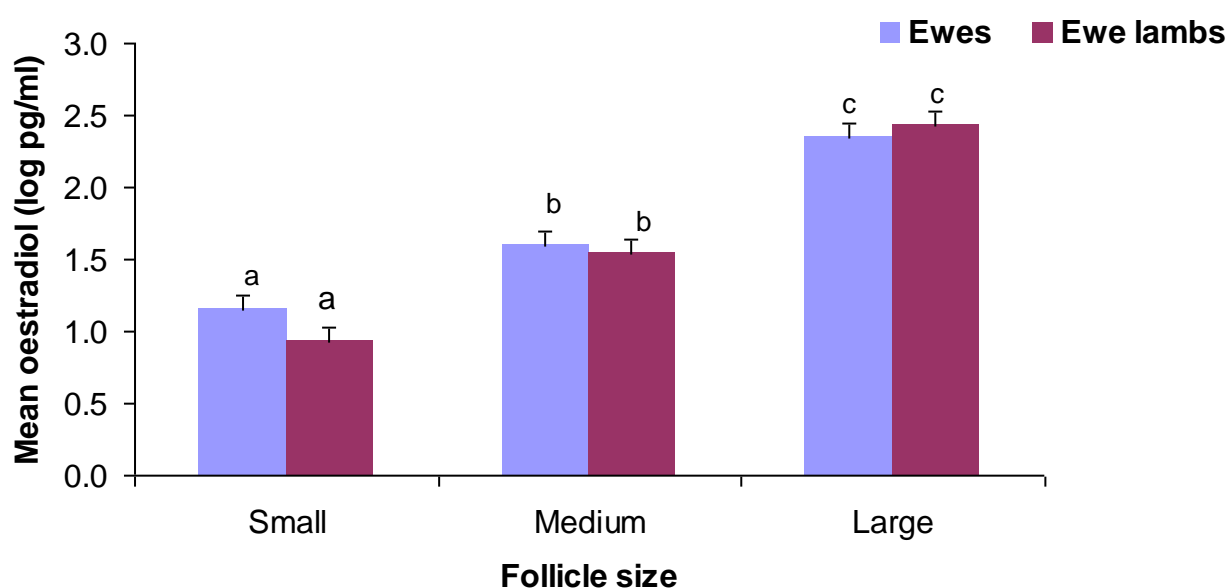


Figure.3.1 The effect of follicle size and animal age on mean concentration of oestradiol produced in culture media after 24 h of incubation (SED = 0.1). Bars with different letters are significantly different, (n = 40 follicles from each size per age).

3.3.1.2 Oestradiol in follicular fluid

The oestradiol concentrations in the follicular fluid of ewes and ewe lambs follicles cultured in TCM-199 for 24 hours are given in Figure.3.2. There were no differences in follicular fluid oestradiol concentration between large follicles from ewes and ewe lambs ($P>0.05$) or between large follicles from ewes and ewe lambs and medium follicles from ewes, ($P>0.05$). However, oestradiol concentrations in medium size follicles of ewes were significantly greater than in medium follicles from ewe lambs ($P<0.05$).

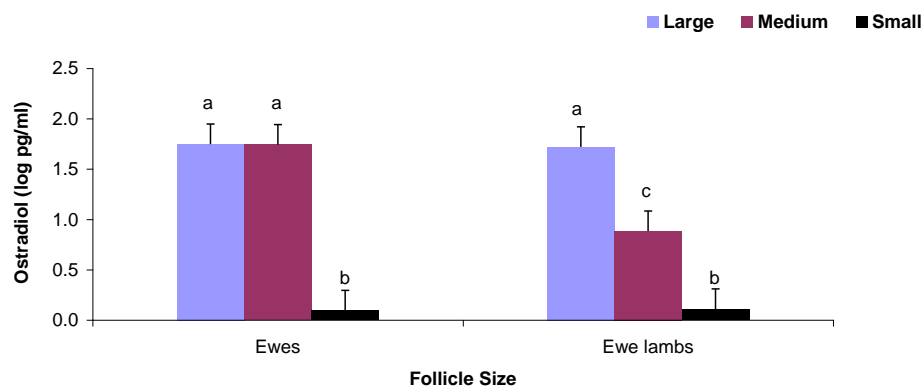


Figure.3.2 The effect of follicle size on mean oestradiol concentration in follicular fluid in ewes and ewe lambs (SED = 0.09). Bars with different letters are significantly different, (n = 15 follicles for each size per age).

3.3.1.3 Follicles cultured for 2, 4, 6 and 8 h in TCM-199

There was no significant difference ($P>0.05$) in oestradiol concentrations after 2, 4, 6 and 8 hours of incubation between ewes and ewe lambs or between follicle size (Figure.3.3 and Appendix.3:1). There was a tendency for oestradiol concentration to decrease with time.

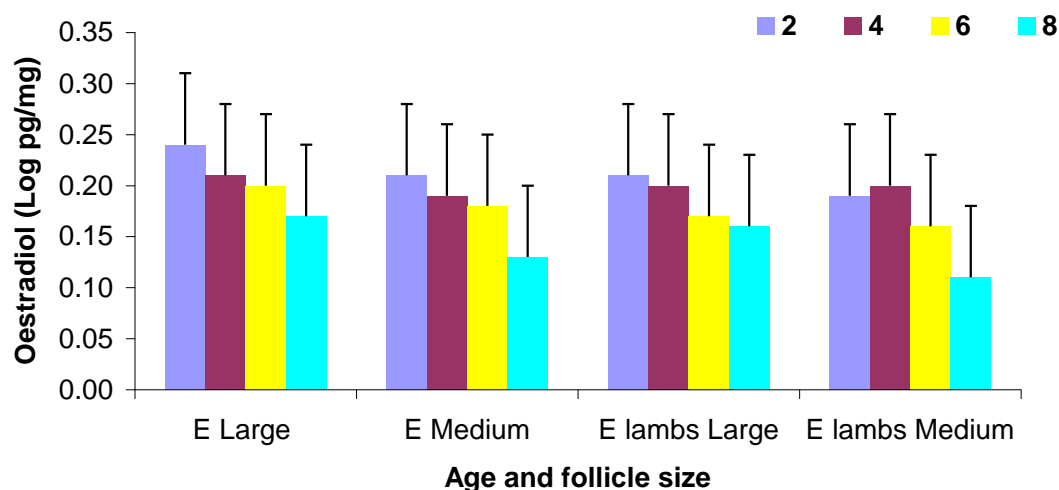


Figure.3.3. The effect of incubation time on mean oestradiol concentrations in ewes and ewe lambs half follicles cultured in TCM-199 (SED = 0.07). E Large: ewe large follicles, E Medium: ewe medium follicles, E Lambs large: ewe lambs large follicles, E lambs Medium: ewe lambs medium follicles (n = 8 follicles for each time per age).

3.3.1.4 Oestradiol in media from follicles cultured for 24 h in TCM-199 with FCS

The results of this treatment revealed that in overall follicles cultured in TCM-199 with FCS produced significantly more ($P<0.05$) oestradiol than TCM-199 without FCS in both ewe and ewe lamb follicles (Mean log oestradiol was 2.44 pg/ml in media with FCS and 2.195 pg/mg in media without FCS, SED= 0.05). No significant ($P>0.05$) difference in oestradiol concentration between ewe and ewe lamb follicles when cultured in TCM-199 with FCS, (Figure.3.4 and appendix.3.2). Mean log oestradiol in ewe follicles media was 2.347 pg/mg, and for ewe lambs was 2.289 pg/mg (SED = 0.05).

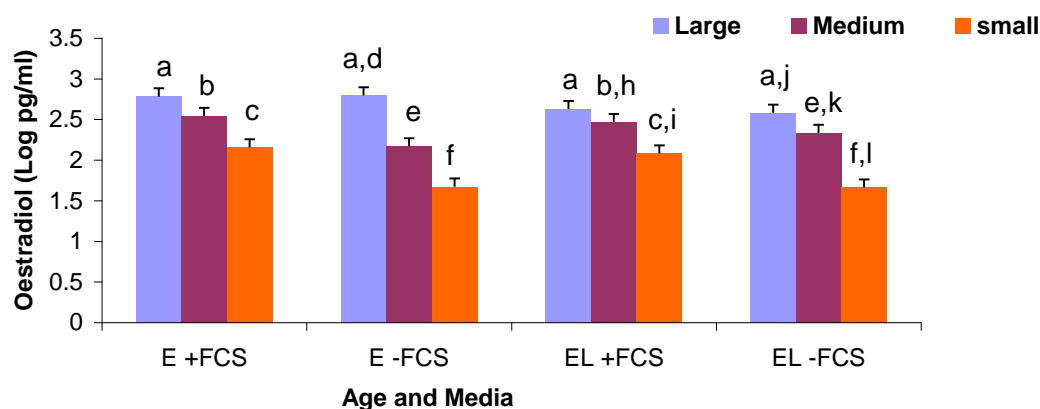


Figure.3.4 The effect of FCS on mean oestradiol concentration in ewes and ewe lambs follicles media (SED = 0.1). Bars with different letters are significantly different. E PFCS: Ewes follicles cultured with fetal calf serum, E MFCS: Ewes without fetal calf serum, EL PFCS: Ewe lambs follicles cultured with fetal calf serum, EL MFCS: ewe lambs follicles cultured without fetal calf serum (n = 10 follicles for each size/medi.pre age).

3.3.1.5 Progesterone concentrations in media of follicles cultured in TCM-199

Progesterone levels in media showed that ewe follicles produced significantly more ($P<0.05$) progesterone than ewe lambs in all size, after culture for 24 hours (Figure.3.5). In ewes, small follicles produced significantly less ($P<0.05$) progesterone than medium and large follicles. In

ewe lambs, progesterone production increased in proportion to follicular size, with medium follicles producing more than small follicles and large follicles producing significantly more ($P<0.05$) progesterone than medium (Figure.3.5 and Appendix.3.3).

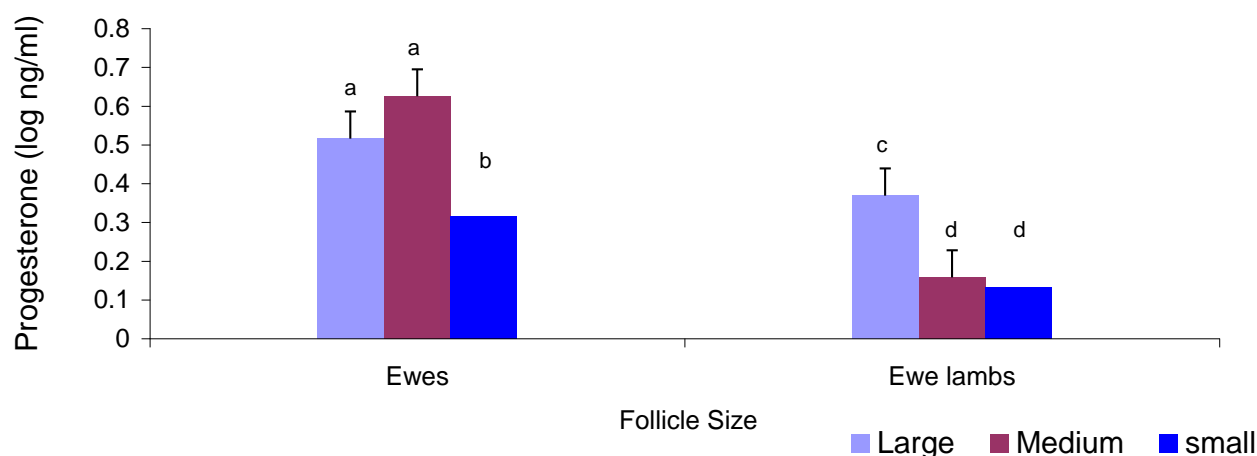


Figure.3.5 The effect of follicle size on mean progesterone concentration in ewes and ewe lambs follicles media after 24 h of incubation (SED = 0.05). Bars with different letters are significantly different ($n = 40$ follicles from each size per age).

3.3.1.6 Progesterone concentration in follicular fluid

A significant difference ($P<0.05$) was found in progesterone concentration in the follicular fluid of different size follicles of ewes and ewe lambs follicles cultured for 24 hours in TCM-199 (Figure.3.6). In ewes, progesterone level was significantly lower ($P<0.05$) in small follicles compared to medium and large follicles. Furthermore, no significant difference ($P>0.05$) in progesterone concentrations was found between medium and large size in ewes. This differed to ewe lambs where medium and small follicles produced significantly less ($P<0.05$) progesterone than large follicles (Figure.3.6). In addition, no difference was observed between

small ewe follicles and small ewe lamb follicular fluid. However, ewe lamb medium follicles produced significantly more ($P<0.05$) progesterone than small ewe follicles (Figure.3.6).

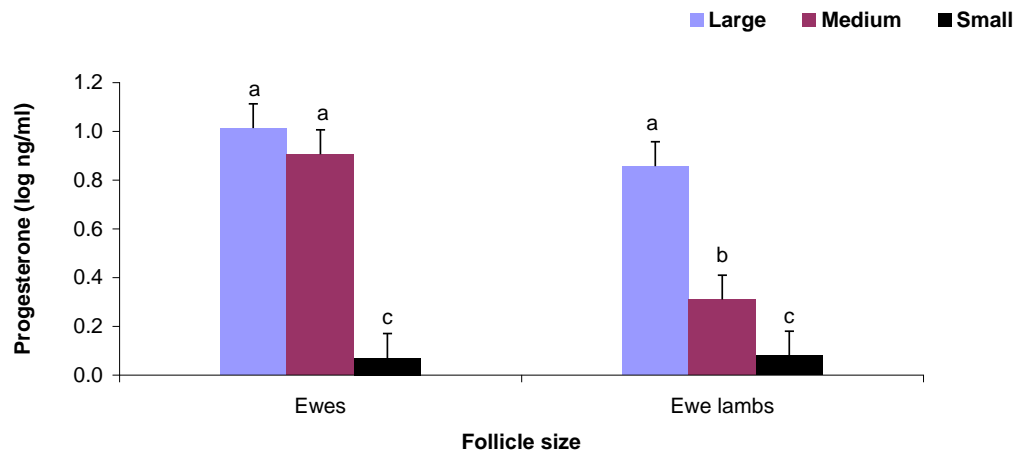


Figure.3.6 The effect of follicle size on mean progesterone concentration in ewes and ewe lambs follicular fluid (SED = 0.18). Bars with different letters are significantly different (n= 15 follicles for each size per age).

3.3.1.5 Progesterone concentration in the media of follicles cultured for 2, 4, 6 and 8 h in TCM-199

In follicles cultured for different times of incubation, after 2, 4, 6 and 8 h of incubation, there was no significant ($P>0.05$) difference in progesterone production from large ewe follicles and medium ewe follicles, nor from large ewe lamb follicles (Figure.3.7). No difference was observed between medium ewe follicles and large ewe lamb follicles within time, in addition no significant difference was ($P>0.05$) shown between large ewe follicles after 2 h and 8 h (Figure.3.7). Moreover, large ewe follicles produced more ($P<0.05$) progesterone at 2 h and 4 h than ewe lambs at the same time. Progesterone declined with increased time of incubation in both age groups. Medium follicles produced less progesterone than large follicles in both ages

of group ($P>0.05$) Overall, ewe follicles produced more progesterone than ewe lambs, (Figure.3.7 and Appendix.3.4).

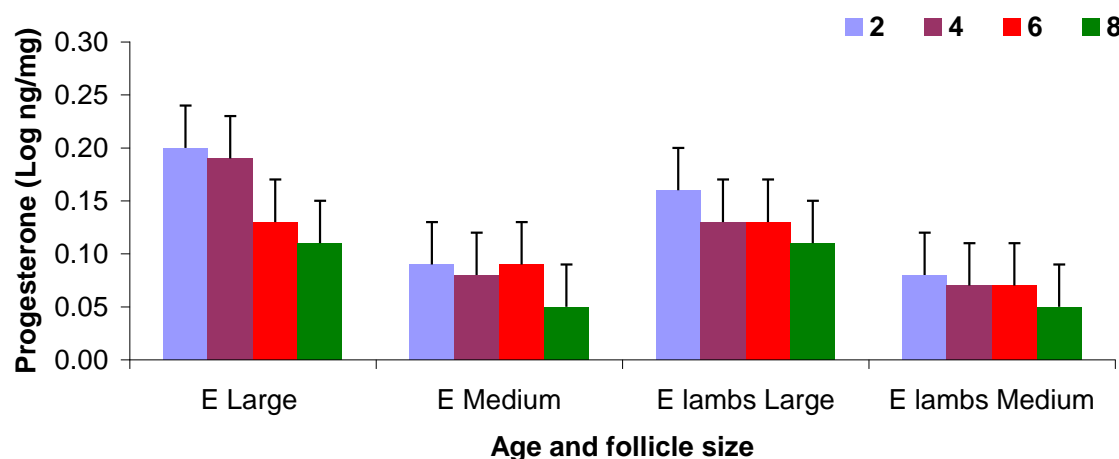


Figure.3.7 The effect of time on mean progesterone concentration in ewes and ewe lambs follicles cultured in TCM-199 (SED = 0.04). E Large: ewe large follicles, E Medium: ewe medium follicles, E Lambs large: ewe lambs large follicles, E lambs Medium: ewe lambs medium follicles. (n = 10 follicles for each size/medi.pre age).

3.3.1.6 Progesterone concentration in media from follicles cultured for 24 h in TCM-199 with FCS
In ewes, large and medium follicles cultured with or without FCS and large ewe lamb follicles cultured with FCS, similar progesterone concentrations were observed (Figure.3.8). Small ewe follicles produced significantly less ($P<0.05$) progesterone than large or medium follicles in both types of media (Figure.3.8). However, no difference was found in ewe lamb progesterone concentration between medium and small follicles in either type of media, but large ewe lamb follicles produced significantly more ($P<0.05$) progesterone than medium or small ewe lamb follicles in media with or without FCS (Figure.3.8). Overall, small and medium ewe follicles

produced more progesterone than medium and small ewe lamb follicles, in both media with or without FCS.

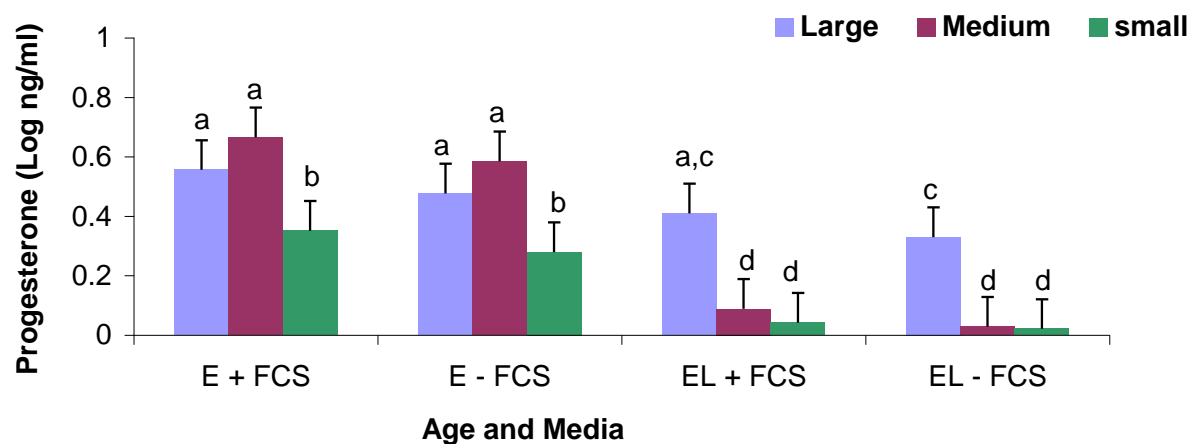


Figure.3.8 The effect of follicle size on mean progesterone concentration in ewes and ewe lambs follicles media with or without FCS (SED = 0.04). Bars with different letters are significantly different. E + FCS: Ewes follicles cultured with fetal calf serum, E -FCS: Ewes without fetal calf serum, EL + FCS: Ewe lambs follicles cultured with fetal calf serum, EL - FCS: ewe lambs follicles cultured without fetal calf serum (n = 10 follicles for each size/medi.pre age).

3.3.2 Corpora lutea progesterone concentration

3.3.2.1 CL cultured for 2 h in TCM-199

Progesterone concentration was significantly higher ($P < 0.05$) in ewes than in ewe lambs at 0 h and after 2 h of CL incubation (Figure.3.9). However, no significant difference was found between times 0 h and 2 h within either age group (Figure.3.9 and Appendix.3:6).

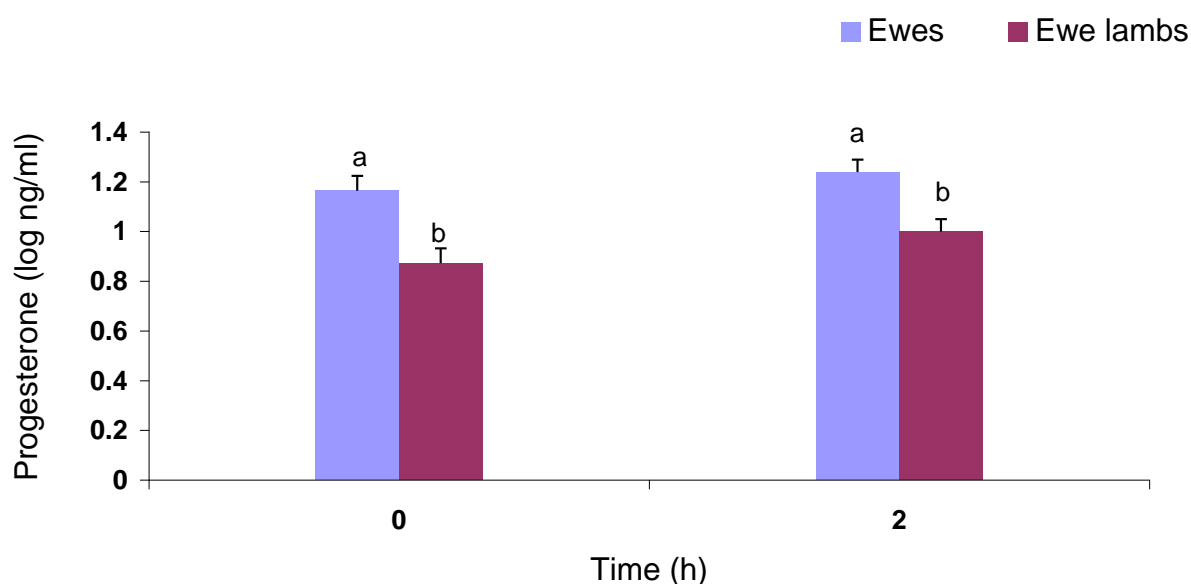


Figure.3.9 The effect of incubation time on mean progesterone concentration in ewes CL (702 mg) and ewe lambs CL (713 mg) cultured for 0 h (SED = 0.06) and 2 h (SED = 0.05). Bars with different letters are significantly different.

3.3.2.2 Progesterone concentrations in the media of CL quarter cultured in four different types of media.

The results revealed that there was no difference ($P>0.05$) between the amount of progesterone produced by ewes and ewe lambs in all media types (Figure.3.10). Within age groups, CL cultured in TCM-99 produced less progesterone than other types of media. The results showed no difference ($P>0.05$) was observed between BSA, FCS and PVA in ewe CL media (Figure 3.10).

In ewe lambs, medium with PVA produced significantly more ($P<0.05$) progesterone than media with BSA and TCM 199 (Figure.3.10 and Appendix.3.6). In addition, in ewe lambs PVA medium produced significantly more ($P<0.05$) progesterone than TCM-199 and TCM-

199 with BSA in both age of groups. Moreover, in ewe lambs, FCS medium produced significantly ($P<0.05$) more than TCM 199.

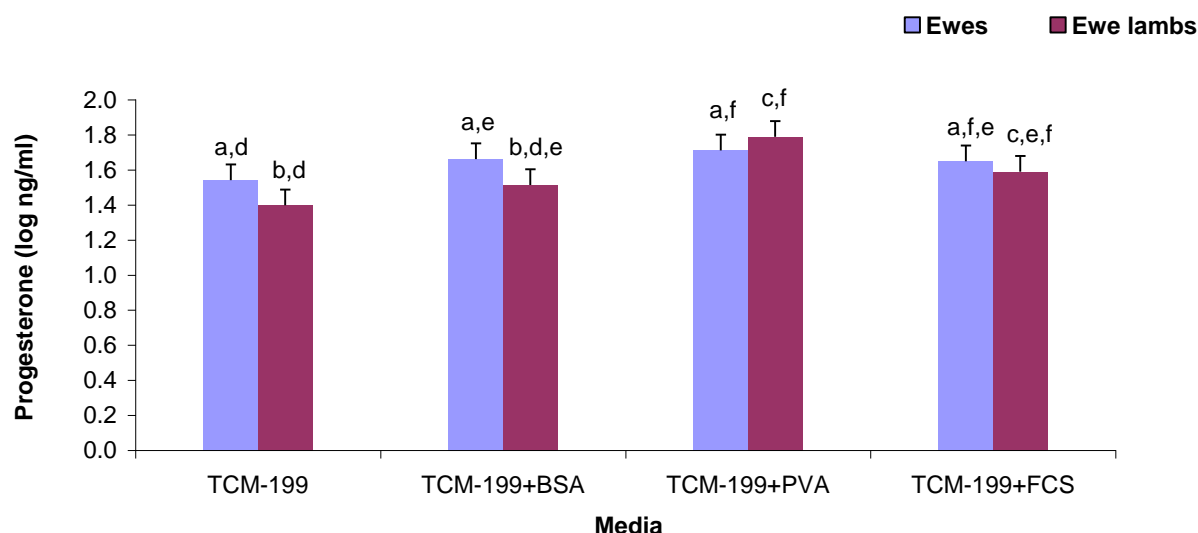


Figure.3.10 Mean progesterone concentration in ewes and lambs CL media cultured in different media for 24 h (SED = 0.09). Bars with different letters are significantly different. TCM-199: tissue culture medium, BSA: bovine serum albumin, PVA: polyvinyl alcohol, FCS: fetal calf serum.

3.3.2.3 Progesterone concentrations in CL tissue cultured in four different media

There was a significant difference ($P<0.05$) in tissue progesterone concentrations between the two age groups. Overall mean log progesterone from ewe tissue was 2.637 ng/ml and from ewe lamb was 2.336 ng/ml (SED = 0.05). However, there was no difference in progesterone concentration in luteal tissue cultured in all types of media within age (Figure.3.11).

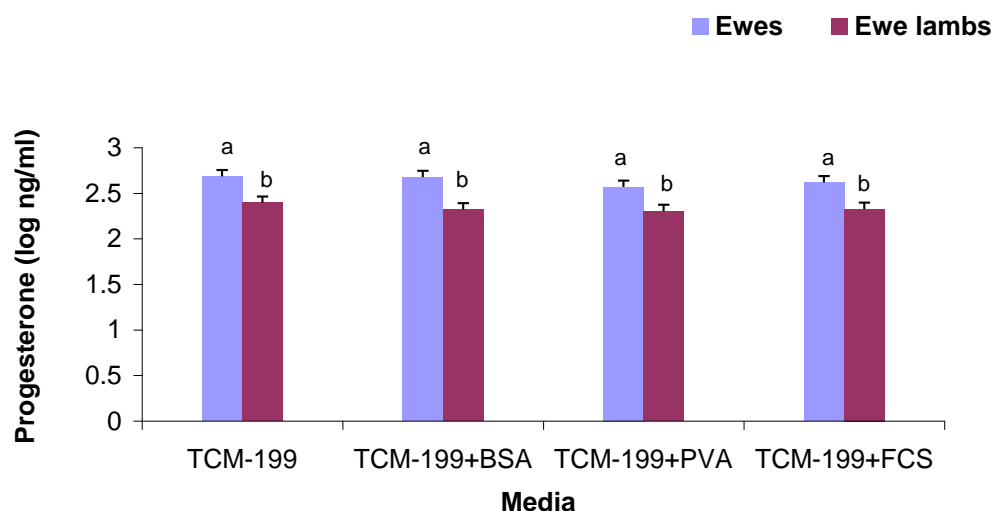


Figure.3.11 The effect of different media on mean progesterone concentration in ewes and ewe lambs luteal tissue after 24 h (SED = 0.03). Bars with different letters are significantly different. TCM-199: tissue culture medium, BSA: bovine serum albumin, PVA polyvinyl alcohol, FCS: fetal calf serum.

3.3.3 Protein electrophoresis analysis

An average gel was created for each age group. The relative % integrated optical density (volume %) was chosen as the best parameter to estimate of protein amount (Barrett et al., 2005). Normalised spot volume was performed as a parameter for test spots. In addition up regulated 2 fold, down regulated 2-fold, the matching percentage, less than 2-fold and the unique spots were taken in account.

3.3.3.1 Protein identification from follicular shells

The results from the comparison data between average (the gel representitive the three repeat gels, also contain the same protein spots in all replicates gels) gels of ewes and ewe lambs follicular shells are shown in Table.3.1. The images of gels were similar. Biological values

were considered to investigate the difference between the two samples, and are presented in Table.3.1. In both gels protein spots are related morphologically. The majority of protein spots in both average gels range from 45 to 97kDa and between *pI* 5 to 8 (Figure. 3.12 A and B). Estimates of the coefficient of variation for relative protein volume range from 22 to 55% for analytical variation, as each gel was made from three replicates of the same sample. A total of 125 spots of ewes reference gel and 202 spots from ewe lambs average gel were counted to compare between the two ages. The two spots of interest are, spot 578 (estimated at 20.1 to 30 kDa marker and *pI* 5-8, showed an increase of 2.07-fold in ewes average gel) and spot 619 (estimated at 14.4 to 20.1 kDa marker and ranging between 5-8 *pI*.) Three dimension images and the montage spots are shown in Figure.3.13 A and B and 3.14. Twenty eight unique spots in ewe average gel compared with 60 spots in ewe lambs average gel were observed (Table.3.1). The percent of identical spots between 3 ewe replicate gels was 67.33 and between ewe lamb replicate gels were 50.5. Progenesis software is very sensitive and picks up every spot and sometimes dust, for that reason it may have counted more number of protein spots in ewe lambs average gel compare with ewes.

Table.3.1 Comparison data between ewe and ewe lamb average gels of medium follicular shell.

Spots	Lambs average gel	Ewe average gel
Total number of spots	155	123
Matched %	47	100
Up regulated 2 fold	8	42
Down regulated 2 fold	42	8
Less than 2 fold	45	45
Unique spots	60	28

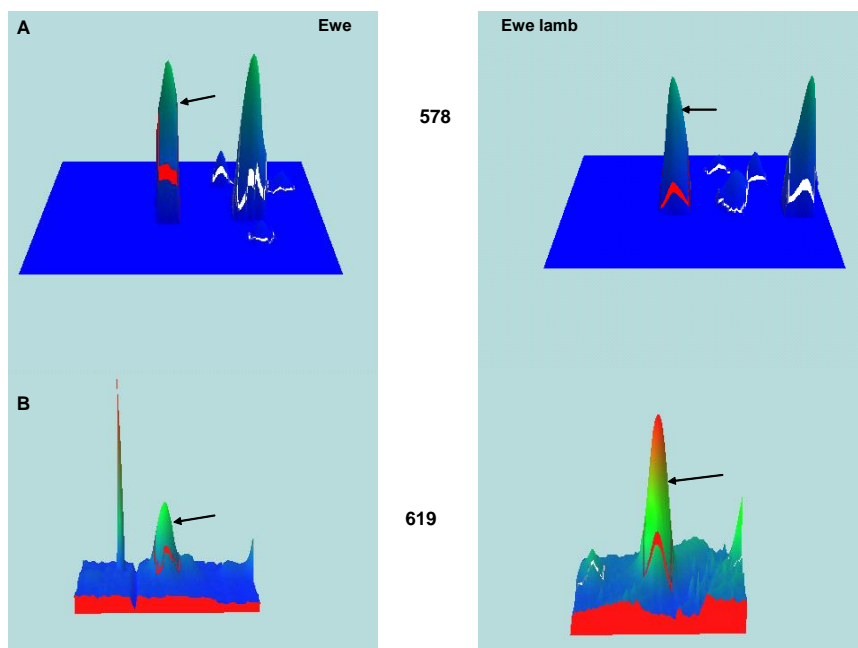


Figure.3.13 Ewes control follicular shells spot (578) up-regulated 2.07-fold (A) and down regulated spot (619) 3.04-fold (B) from ewe lambs average gel.

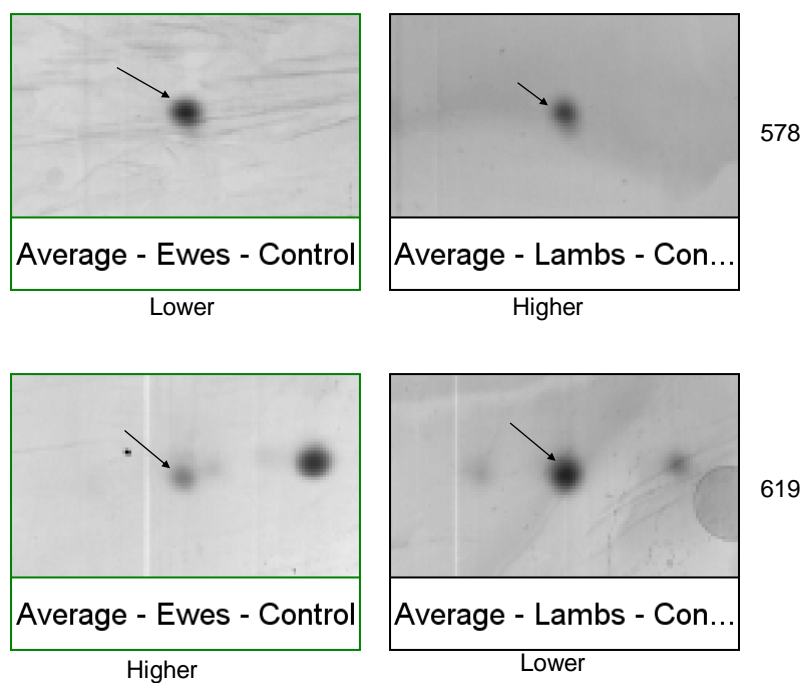


Figure.3.14 Montage images of up and down regulated spots (578 and 619) from ewe and ewe lamb follicular shell average gels.

3.3.3.2 Ewes and ewe lambs luteal tissue electrophoresis

The average gels data of this experiment are shown in Table.3.2. The distribution of protein spots on both gels is quite similar. Average gels in both age groups appeared to contain a large amount of protein spots of high molecular weight (MW) ranging from 20.1 to 100 kDa (Figure.3.15 A and B). A total of 244 and 256 spots were decolonised in ewes and ewe lambs average gels respectively (Table.3.2). The ewe average gel contained fewer up-regulated spots (42) compared with ewe lamb average gel (54), as well as unique spots and more down regulated spots than those found in ewe lambs average gel and unique spots. The number of spots seems to decrease with decreasing of MW. The percent similarity between the 3 ewe replicate gels was 77.18 and between ewe lamb replicate gels was 55.59. In the ewe average gel protein spot 578 showed an increase 2.07-fold, whereas spot 619 showed a decrease 3.04, both spots were estimated at 14.4 to 45.0 kDa marker and ranging between pH 3 to 10 (Figure. 3.16 A and B) and montage spot images are shown in Figure.3.17.

Table.3.2.A Comparison data between ewe and ewe lamb average gels of luteal tissue.

	Lambs CL	Ewes CL
Total number of spots	256	244
Matched %	68	100
Up regulated 2 fold	54	42
Down regulated 2 fold	43	53
Less or up than 2 fold	80	81
Unique spots	79	68

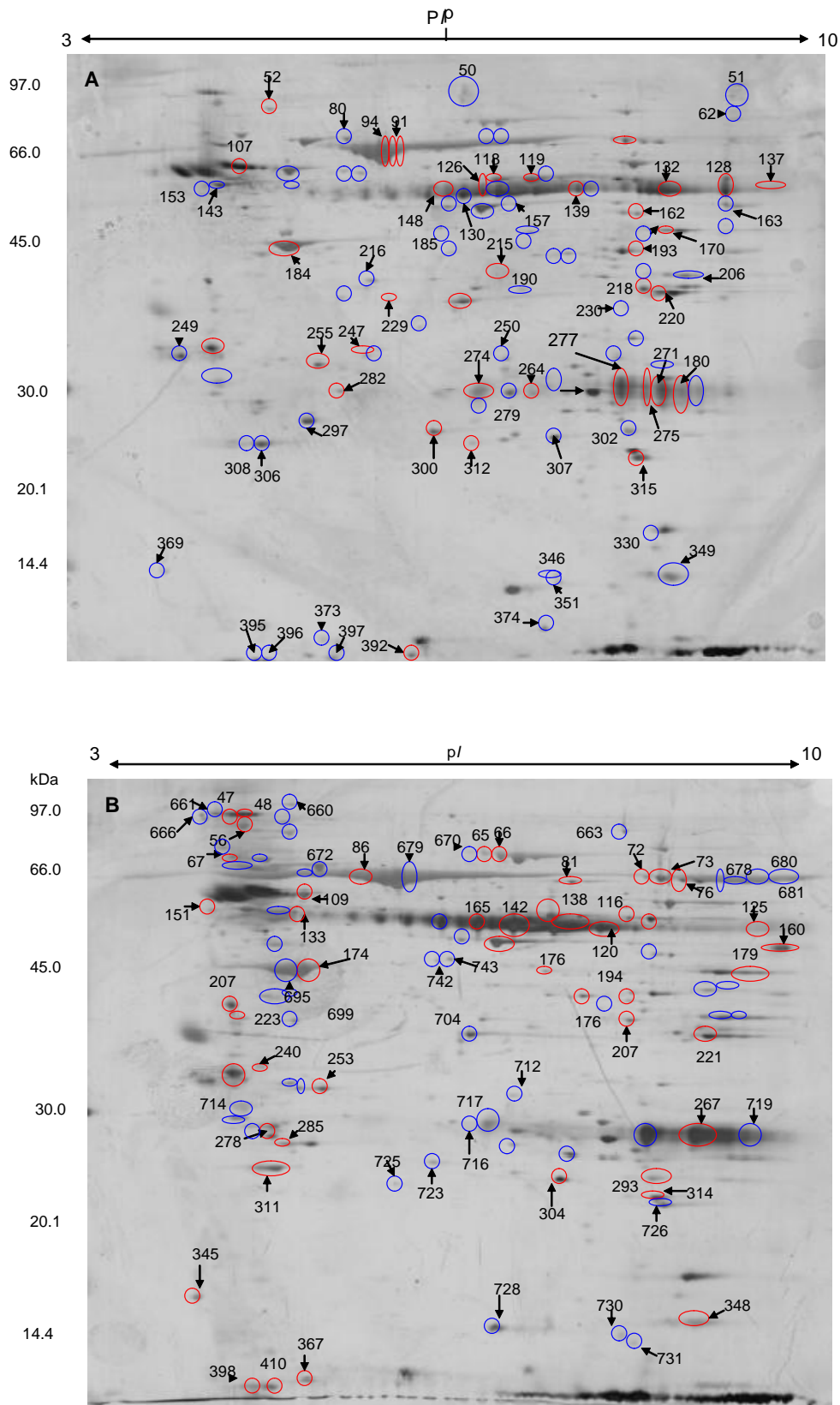


Figure.3.15 100 μ g/ml from ewe average gel from luteal tissue protein (A) and the similar average gel from ewe lamb luteal tissue (red spots) and unique spots (blue). Spots with out circle are less than 2-fold.

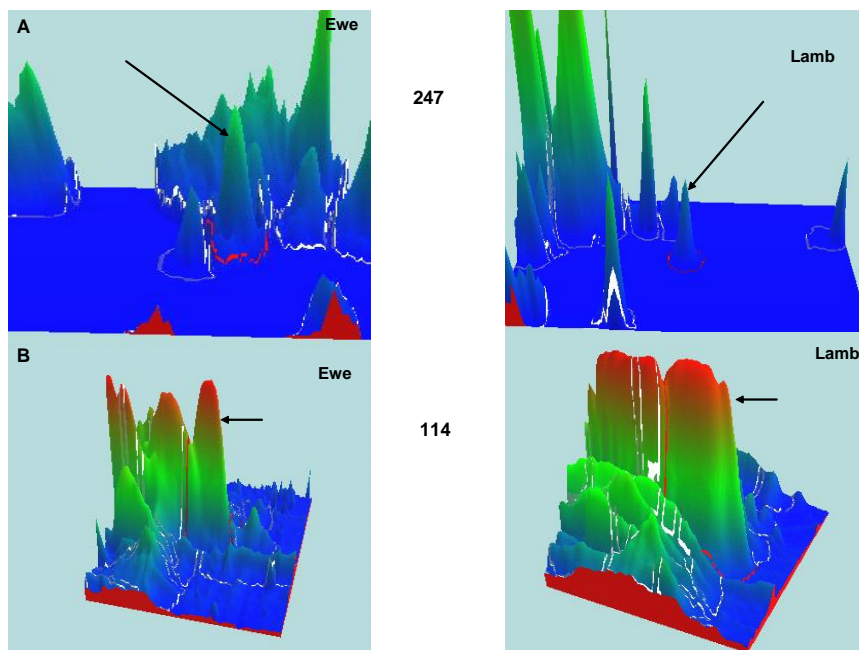


Figure.3.16 A Three dimension images of no: 578, showing a 2.07-fold up-regulation and the same protein spot from the ewe lamb luteal tissue average gels

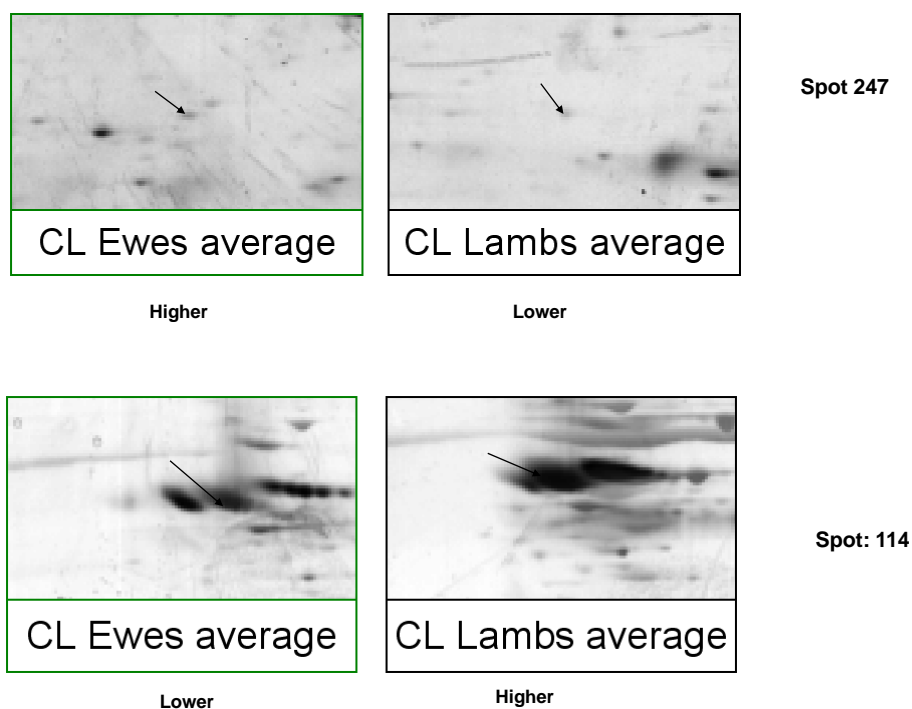


Figure.3.17 Montage images of up and down regulated spots of ewe and ewe lambs luteal tissue protein spots.

3.4 Discussion

Oestradiol concentrations were determined in ewe and ewe lamb follicles media after culture in TCM-199 (Figure.3.1). These findings are in agreement with the results reported for follicle culture in calves and adult cows (Driancourt *et al.*, 2001a) and the basal oestradiol concentrations in the plasma of ewes and ewe lambs *in vivo* where concentrations were similar in both age groups (Khan *et al.*, 2007). This could be due to the activity of enzymes that control the conversion of cholesterol to oestradiol in granulosa cells being similar in ewe lambs and ewes (McCracken *et al.*, 1971).

In addition, the results gained here indicate that in both ewes and ewe lambs, oestradiol concentration in media and follicular fluid increased with follicle size (Figure.3.1). These findings are similar to the results demonstrated by Beck *et al.*, (2003), who reported that in mares follicular fluid oestradiol concentrations were highest in large follicles and lowest in small follicles. This difference is due to the increase in the number of cells in granulosa and theca layers with an increase in follicle diameter. These results are also in agreement with Valasi *et al.*, (2007) who reported that in ewe lambs treated with gonadotrophin, oestradiol concentration was increased with an increase in the size of the follicle for oestrogenic follicles.

The results from oestradiol concentration in follicular fluid indicate that there was no difference between large and medium follicles in ewes and between both these sizes in ewes and large follicles from ewe lambs (Figure.3.2). However, in ewes a highly significant difference in concentration was found between both large and medium and small follicular fluid (Figure.3.2). However, for technical reasons the follicular fluid from each size of follicle (small, medium and large) was diluted in 0.5 ml of PBS and the results do not take this into account. Therefore, a correction should have been made to the concentration to account for

differences in follicle size. Furthermore, the results from this study on follicular fluid oestradiol concentration suggest that the albumin in follicular fluid binds the hormone, because oestradiol concentration is greater in follicular fluid, compared with the low oestradiol concentrations found in the medium after culture. Davies *et al.* (1992), showed that oestradiol secretion *in vivo* was some 20 to 30 fold higher compared with 2 h of *in vitro* incubation. Furthermore, oestradiol concentration was higher in ewes oestrogenic follicles found in the early and mid follicular phase than in the luteal phase or late follicular phase (Baird, 1978).

There was a significant difference in the amount of oestradiol secreted from different follicle sizes. Mann *et al.* (1992), found a positive correlation between follicle size and inhibin secretion after 2 h of incubation. A large amount of oestradiol was secreted from large pre-ovulatory follicles and a small amount was secreted from small follicle with a large amount of inhibin (Mann *et al.*, 1992). Moreover, Moor *et al.* (1973), mentioned that gonadotrophins were found in follicular fluid, that could consequently provide a source of hormonal stimulation for follicles *in vitro*. In this experiment, gonadotrophins might be present in ewes follicular fluid more than ewe lambs follicular fluid, as granulosa cells have to be exposed to LH (Channing, 1970). In contrast Gonzalez-Bulnes *et al.*, (2004) said that oestradiol secretion increase is related to the size of the pre-ovulatory follicle at first phase of development. Hay and Moor (1973), reported that the capacity of cultured follicles to secrete oestrogen and progesterone is not affected by the removal of the follicular fluid and this level of hormone secretion can remain stable after reaching a threshold.

The results for oestradiol after 24 h incubation may not reflect their physiological function as the tissue may differentiate over time. Therefore, the second part of this experiment was

designed to examine the oestradiol concentrations after different times of incubation using the same medium. The results from this experiment demonstrate that there was no difference found in oestradiol concentrations in different times of incubation between ewes and ewe lambs in media of cultured follicles (Figure.3.3). The media was changed every 2 h and the level of hormone found during incubation time indicated the tissue was still living and producing hormone.

A significant difference was observed in oestradiol concentrations when medium with FCS was compared to medium without FCS (Figure.3.4). The results also indicated that oestradiol was higher in ewes than ewe lambs in medium with FCS, indicating the response of follicular tissue to FCS components, such as growth factors and protein which stimulate oestradiol secretion. Moreover, oestradiol concentration increased with the larger follicle size in both age groups. These findings are similar to results found in the first experiment when follicles were cultured in TCM-199, and is also in agreement with the results reported by Valasi *et al* (2007). FCS is often used to culture bovine (Katska and Rynska, 1998, Saha *et al.*, 2000) and ovine follicles (Cecconi *et al.*, 1999). Fetal calf serum is used as protein supplement for follicles in this culture. The results suggest that fetal calf serum has a positive effect on cultured follicles in this experiment, stimulating oestradiol production in maturation media, due to the growth factors in FCS. Furthermore, those ewe follicles are more responsive to FCS than those from ewe lambs.

The data from follicles cultured in TCM-199 for 24 h showed no difference in progesterone concentration was found between medium and large size ewe follicles (Figure.3.5). However, large and medium ewe follicles produced significantly more progesterone than those in ewe

lambs. The difference between the two age groups may be due to a difference in the process of cholesterol conversion to progesterone and the enzymes of this process. Also, ewe lambs need cholesterol for growing energy, for this reason a lesser amount of cholesterol will be available to be converted to progesterone. The number of granulosa cells is less in ewe lambs compared with those in ewes and therefore the amount of cholesterol changed to progesterone is less in ewe lambs than in ewes. In ewe lambs, progesterone levels gradually decreased with a decrease in follicle size, and a significant difference was observed between large size follicles compared with medium and small, although no difference was found between medium and small. This result is similar to the result found by Valasi *et al.*(2007).

Progesterone concentrations in follicular fluid increased with an increased follicle size in both age groups (Figure.3.6). Although there were no differences between large and medium size follicles in ewes, a significant difference was observed between large and medium follicles in ewe lambs. However, in both ages, small sized follicles produced significantly less progesterone than either medium or large (Figure.3.6). The follicular fluid from each size of follicles (small, medium and large) was for technical reasons diluted in 0.5 ml of PBS. The results did not take this into account. Therefore, that data is uncorrected for dilution. In ewes treated with PMSG during the oestrous cycle, *in vitro* progesterone secretion was ten times higher than that produced from cultured follicles of untreated ewes at the same stage (Moor *et al.*, 1973). These patterns of hormone secretion have also been shown in cultured cattle follicles, where no difference was found in steroid production when cultured on grids or rolling tubes (Kruip and Dieleman, 1989). These findings suggest that bovine steroidogenic follicle function depends on external factors like culture system and culture time, as well as internal factors such as follicle size, stage of cycle and quality of follicles (Kruip and Dieleman, 1989).

The results of progesterone from cultured follicles in TCM-199 for different incubation times demonstrate that in ewes, medium follicles produced less progesterone than large follicles within the same time (Figure.3.7). In large ewe follicles, the only significant difference was found after 2 h and 8 h of incubation. However, the difference between medium and large ewe follicles was observed at 2 h and 4 h of incubation and in ewe lambs the results indicate that progesterone concentration was higher in large follicles than medium follicles, with a significant increase noted after 4 h, 6 h and 8 h of incubation. Overall, ewe follicles produced more progesterone than ewe lambs in both sizes of follicles. In addition, follicles were still viable after 8 hours. These results are in contrast with those reported by Gonzalez-Anover *et al.* (2007) who showed that *in vivo*, steroid hormones secretion from follicles of Negra de Colmenar ewes, were not affected by follicle size. However, other studies have reported the predominance of androgens in the follicular fluid of small and medium follicles is expressed in the predominantly androgenic profile of steroids produced by the same size of follicles in the static rolling tube system (Henderson *et al.*, 1982, Kruip and Dieleman, 1985, Kruip *et al.*, 1979). It is postulated that, the steroid concentration in the follicular fluid reflect the steroidogenic activity of the follicles but not the steroidogenic capacity (Kruip and Dieleman, 1989).

The results of progesterone from follicles cultured in TCM-199 with FCS revealed that in ewe and ewe lambs, follicles cultured in medium with FCS produced more progesterone than medium without FCS (Figure.3.8). In ewes no significant difference was observed between large and medium follicles when cultured with or without FCS. However, significantly less progesterone was observed in small follicles compared with medium and large follicles in both types of media. Also in ewe lambs, the large follicles produced more progesterone than

medium and small follicles in both types of media (Figure.3.8). Overall, FCS stimulated greater progesterone production, possibly due to the factors in contains e.g nutrients, proteins, growth factors, hormones and cholesterol.

The results of cultured luteal tissue for 2 h in TCM-199 showed that progesterone concentration after 0 and 2 h of incubation was significantly higher in ewes CL and media than in ewe lambs (Figure.3.9), even though there was no difference in the tissue weight between ewes and ewe lambs (ewes 702 mg and ewe lambs was 713 mg, SED = 41.9). This could be explained by LH receptors being in higher abundance in ewe luteal tissue than in ewe lambs luteal tissue at the same time of the oestrous cycle. This inadequacy of ewe lambs luteal LH receptors and the lower amount of progesterone secreted compared with adult ewes, could be one reason responsible for ewe lambs subfertility. In addition, ewe luteal tissue may include a greater number of secretory luteal cells than ewe lambs tissue, therefore, producing more progesterone than ewe lambs tissue.

The results for progesterone concentration revealed that in ewes, there was no difference found for CL cultured in different types of media (Figure.3.10). Moreover, there was no difference between ewes and ewe lambs with media, which could mean that after 2 h of incubation luteal tissue did not responded to the culture media, because for tissue concentrations a significant difference was found between the ages of animals. However, the unexpected significant difference in ewe lambs TCM-199 plus PVA medium and TCM-199 with BSA, and between TCM-199 and FCS could be due to experimental error such as tissue degradation Figure.3.10.

Progesterone concentration in cultured luteal tissue data showed was no difference found within age group between the four types of media. However, a significant difference ($P < 0.05$) in progesterone was found between age groups for each media (Figure.3.11). These results could be explained as ewes luteal tissue includes a higher number of luteal cells, which are responsible for hormone production compared with ewe lambs. Furthermore, the secretory tissue in ewe lambs CL may not develop as much as in adult ewes, therefore, the response to culture media would be less and the amount of hormone would be lower in ewe lambs compared with ewes. This inadequacy of ewe lambs luteal function might explain the higher embryonic mortality in ewe lambs and consequently poor reproductive performance in ewe lambs, which leads to the use of progestagen and PMSG in vivo to improve it (Quirke, 1979b). In addition these results could be explained by the luteal tissue in ewes containing more progesterone than ewe lamb tissue, but it was not released it into media as no difference was found between age groups in media progesterone concentrations. It was unclear from the results of the hormone assays how the differences between age groups in hormone secretion were caused. Therefore the study of protein expression was undertaken using the same amount of homologous tissues from both ages of groups as used in the hormones studies, in order to determine differences between the two ages.

The results from protein analysis of follicular shells showed more protein spots present in the ewe lamb average gel (202) compare with ewe average gel (125). Progenesis software is extremely sensitive when using the automated detects spots function. There was in replicate gels, not only are true protein spots were detected, other gel imperfections such as dust and air bubbles maybe included in the analysis. To eliminate such contaminates each replicate gel requires individual manual manipulation in order to identify only the spots. Unfortunately this

could not be completed due to time limitation. However, their imperfections are eliminated by averaging the gels.

The number of up-regulated 2-fold protein spots were higher (42) in ewe than in ewe lamb (8) with low a matching percent in the ewe lamb average gel. These results could explain that the enzymes in the theca and granulosa cells of ewe shells are more active than those in ewe lamb, which causes the secretion of more oestradiol and progesterone in ewes compared with ewe lambs. However, the molecular weight range of where most large protein spots were detected was similar in both age groups (30 to 97kDa marker).

Results from luteal tissue protein analysis revealed that in both ewes and ewe lambs average gels were similar, with 68% matching. The total number of protein spots was 244 in the ewe average compared with 256 spots in ewe lamb average gel. In both average gels the abundance protein spots were estimated between 14.4 to 97 kDa with varying protein spots between the two ages. This result reflects the similarity between the age groups. In this regard this study required further more investigation to contribute in improvement of ewe lambs reproductive performance.

3.5 Conclusion

The results of this experiment demonstrate that overall ewe follicles produced more oestradiol and progesterone than ewe lamb follicles when cultured in TCM-199 under the same culture condition. Also the large size produced more hormones than small size and ewe follicles media secreted more hormones in FCS medium than ewe lamb follicles media. Ewe luteal tissue produced more progesterone than ewe lamb luteal tissue when cultured in different culture

media Moreover, proteomics analysis showed an increase in ewe protein spots compared with ewe lamb gels in both follicular shells and luteal tissue gels.

Chapter Four

**A comparison of hormone stimulated follicular/luteal
steroidogenesis *in vitro*, and follicular proteomics in ewes and ewe
lambs**

4. 1 Introduction

Khan *et al.* (2007) demonstrated that ewe lambs were less responsive to gonadotrophin challenge than ewes. In contrast to ewes, plasma oestradiol concentrations remained unchanged in lambs following GnRH or hCG treatment. Furthermore, the increase in plasma progesterone concentrations following GnRH or hCG were significantly lower in ewe lambs than in ewes. This suggests that follicular and luteal tissue in ewe lambs is less able to respond to gonadotrophin stimulation. Although, other factors, previously discussed in chapter 3, may be responsible for the observed differences in plasma steroid concentration.

Therefore, this experiment was designed to determine whether follicular and luteal tissue of ewes and ewe lambs respond differently gonadotrophin *in vitro*. In addition, to determine whether the *in vitro* stimulation of ewe and ewe lamb follicles results in different patterns of protein synthesis this may be associated with differences in steroidogenic response.

4. 2 Materials and methods

4. 2. 1 Experiment 1: To compare oestradiol and progesterone secretion in ewe and ewe lamb follicles cultured in vitro in TCM199 with different concentrations of gonadotrophins

4.2.1.1 Experimental 1a: Follicles cultured in one concentration of FSH/LH

Ewes Welsh Mountain, Black face and Speckle breeds at five years old (n = 99) and ewe lambs (Texel cross Mule) at 7-8 months (n = 99) were used in this experiment. The average weight was 46.42 and 33.46 kg (SED = 0.68 and 0.35) for ewes and ewe lambs respectively. Condition scores were individually recorded and averaged 2.59 and 2.52 (SED = 0.056 and 0.050) for ewes and ewe lambs respectively. Animal breeds, weight and condition score are shown in Appendix.2.B. Ewes were separated into two pens. One raddled ram (Lleyn breed) was

introduced to each pen. Ewe lambs were divided into three groups. The first group consisted of 34 ewe lambs and the other two groups consisted of 33. One raddled ram was introduced into each group for oestrus detection. Animal management and nutrition were as described in section 2.3.

All animals were slaughtered in groups at day 9-12 after oestrus. Frequently two groups of animals, one from each age group, were slaughtered together. Ovary collection, follicle dissection and classification were done as described in section 2.4. and 2.4.1. All follicles were grouped according to size (small, medium and large) and washed three times in TCM-199 with antibiotics. From each size group, 40 follicles were randomly chosen from both ewes and ewe lambs and individually placed into 24 multiwell dishes. Follicles were cultured whole in 2 ml TCM-199 containing 10 µg FSH and 20 µg of LH (treatment) or 40 µl of sterilized PBS (control), and incubated at 37°C and 5% CO₂ for 24 hours. After incubation, media were collected separately and stored at -20°C until oestradiol and progesterone were assayed as described in section 2.5.1 and 2.5.2. The follicles were stored separately at - 80°C for proteomics.

4.2.1.2 Experiment 1b: Follicles cultured in different concentrations of FSH/LH

Ewes (n = 9) and ewe lambs (n = 18), (age and breeds as described in section 4.2.1.1), were used for this experiment. Follicles were collected as described above using sterile forceps and a scalpel blade, large follicles from each age were randomly selected and cut into four quarters. All quarters were placed into a petri dish (100 x 20 mm) and gently washed three times in pre-warmed TCM-199 with antibiotics. Using sterile forceps each quarter was placed into one well of a 24 multiwell dish and then cultured in 2ml of TCM-199 with antibiotics plus one of four

different concentrations (0/0; 0.005/0.05; 0.05/0.5 and 0.5/5 µg/ml of FSH/LH) and incubated at 37°C and 5% CO₂. Media were collected separately after 2, 4 and 6 h and kept at - 20°C for hormone analysis. Oestradiol and progesterone were analyzed by RIA as described in section 2.5.1 and 2.5.2.

4.2.2 Experiment 2: To compare progesterone secretion in ewe and ewe lamb corpora lutea cultured in TCM-199 with different concentrations of LH or hCG

This experiment was designed to culture luteal tissue of ewe and ewe lamb in different amount of LH or hCG in different time of incubation

4.2.2.1 Experiment 2a: CL cultured in four different concentrations of LH for 24 hours

The ovaries were used in this section are the same as those used in section 2.4.1.1. CL s (n = 30 from each age of animals) were dissected out of the ovaries, gently cleaned of adipose tissue and washed as described in section 2.4.2. With sterile forceps and a scalpel blade, each CL was divided into four equal pieces. All pieces were weighed, then each quarter was minced with sterile forceps and scalpel blade and then cultured in 2 ml of pre warmed TCM-199 with one of the following concentrations of LH (0, 10, 100 and 1000 µg/ml) and incubated at 37°C and 5% CO₂ for 24 hours. Media from each quarter was collected separately and stored at - 20°C. The average weight of the four pieces was for ewes 167.2 ± 57.69 , 160.53 ± 45.96 , 162.3 ± 43.81 and $167.9 \text{ mg} \pm 49.95$ and for ewe lambs was 164.67 ± 60.60 , 160.33 ± 62.43 , 165.5 ± 52.34 and 179.33 ± 66.03 .

Progesterone was extracted from each piece with petroleum ether prior to assay. Each CL piece was homogenized carefully with 2ml of PBS in ground glass homogeniser (Fisher Scientific, UK) and 3 ml of petroleum ether was added to each sample before being filtered. The homogeniser and filter paper were washed by 2 ml of diethyl ether, vortexed gently for 30 min,

centrifuged for 10 minutes at 3000 rpm and then allowed to equilibrate for 1 hour at - 20°C. The tubes were placed on an evaporative system until completely dried in the fume cupboard. The solvent residue was redissolved in 3 ml of progesterone buffer assay and shaken gently before being transferred back to the former tube and incubated over night at 4°C. Before the samples were assayed all CL tissue extract samples were diluted 1: 100 and media sample were diluted 1:10 with progesterone buffer assay. Progesterone was estimated by RIA as described in section 2.5.1.

4.2.2.2 Experiment 2b: CL cultured in four different concentrations of LH for 2, 4 and 6 hours

Corpora lutea were dissected out from ovaries described in section 3.2.1.1. The collection process was as described in section 2.4.2. Fifteen CLs from each age group were weighed and divided into two halves, one half was immediately frozen at – 80°C for electrophoresis. The other half was cut into four pieces, each piece was gently minced using sterile forceps and scalpel blade and then washed three times with warmed TCM-199 (33°C). All four pieces from the same CL were cultured in 2 ml of TCM-199 with one of the four different dilutions of LH (0, 10, 100 and 1000 ng/ml) and incubated as described in section 4.2.2.1.1. The average weights of CL piece for each treatment were 67.22, 67.62, 68.36 and 74.28 mg (SED = 14.50, 18.47, 15.66 and 16.98) for ewes and 49.96, 62.38, 62.45 and 67.34 mg (SED = 12.19, 20.03, 21.27 and 16.58) for ewe lambs at 0, 10, 100, 1000 ng/ml LH concentration respectively. Media was collected and refreshed after 2, 4 and 6 h of incubation. The media were stored at - 20°C until progesterone was assayed. All samples were diluted 1: 100 using progesterone buffer assay and progesterone measured by RIA as described in section 2.5.1.

4.2.2.3 Experiment 2c: CL cultured in four different concentrations of hCG for 2 and 4 hours

Corpora lutea were collected from ovaries as described in section 2. 4.1.2 and dealt with as described in section 2.4.2 from ewes and ewe lambs slaughtered between day 9 to day 12 of oestrus. Corpora lutea were weighed after being washed three times in TCM-199 with antibiotics and cut into approximately four pieces. The average weight of the pieces were 160.94, 202.91, 180.51 and 198.58 (SE= 63.76, 66.69, 55. 72 and 54.73) for ewes and 129.14, 219.73, 243.48 and 216.08 (SE = 70.89, 80.95, 114.60 and 107.18) for ewe lambs. All the pieces were minced by sterile forceps and scalpel blade. All four pieces (from the same CL) were cultured in 2 ml of pre-warmed TCM-199 and antibiotics plus one of four dilutions of hCG (0, 0.1, 1 and 10 iu/ml). The tissues were incubated as described in section 2.4.2.1.1. The media was collected and replaced after 2 and 4 h and stored at -20°C until the progesterone assay. All the samples were diluted 1:100 in progesterone buffer assay. Progesterone was determined as described in section 2.5.1.

4 2.3 Proteomics of follicular shells

Follicular shells dissected as described in section 4.2.1.1, were used in this experiment. Two groups of follicular shells were used for each age of animal. Within the age group, gels were done for each follicle size (12 small, 6 medium and 12 large follicular shells) were used for treatment and control follicles. Were no Gel replicated due to the problems with gel quality. Sample preparation was done as described in section 3.2.3.1.1 and the whole process of electrophoresis was conducted as described in section 3.2.3.1.2.

4.2.4 Statistical analysis

All statistical analyses were carried out using the GenStat statistical package. Data from experiment 1, one concentration of gonadotrophin were analysed by general analysis of

variance, whereas data from different concentrations of gonadotrophin and different concentrations of hCG by split-plot design. Furthermore data from the CL cultured for 24 h (Experiment 2a) in different amount of LH were analysed, by general analysis of variance with number of animal as (ID) block. Data from cultured CL in different concentrations LH or hCG for different incubation times were analysed by split-plot with number of animal as (ID) block. The difference between all individual means was determined by the s.e.d All data were transformed using the log transformation. Proteomics data were analyzed using Progenesis software as described in secretion (2.7.4), the comparison was done using one treated gel verses one control gel from the same follicular shells and for the same age of age group. Biological value was considered in analyzing gels, like up-regulated 2-fold down regulated 2-fold and unique spots.

4.3 Results

4.3.1 Experiment 1a: Follicles cultured in TCM199 with or without FSH/LH for 24 h

4.3.1.1 Oestradiol

Overall, follicles cultured with gonadotrophin produced more oestradiol than control follicles in both ewes and ewe lambs follicles (Mean of log oestradiol concentration in treatment was 1.759 pg/ml and control concentrations was 1.572, SED = 0.064).

A significant difference ($P < 0.05$) was observed between follicles size. Oestradiol concentration in media from large follicles was more than that produced from medium and small follicles, and media concentrations from medium follicles greater than that of the small follicles, in both age of groups (Figure.4.1). However, no significant ($P > 0.05$) difference was also observed between the two ages. Small treated follicles from ewes produced more oestradiol than small

control ewe lambs follicles, and control medium ewe lamb follicles produced less oestradiol than medium treated follicles from ewes and ewe lamb (Figure.4.1). Oestradiol concentrations were higher in media from ewe follicles than media from ewe lamb follicles, in both treatment and control groups (Appendix.4.1).

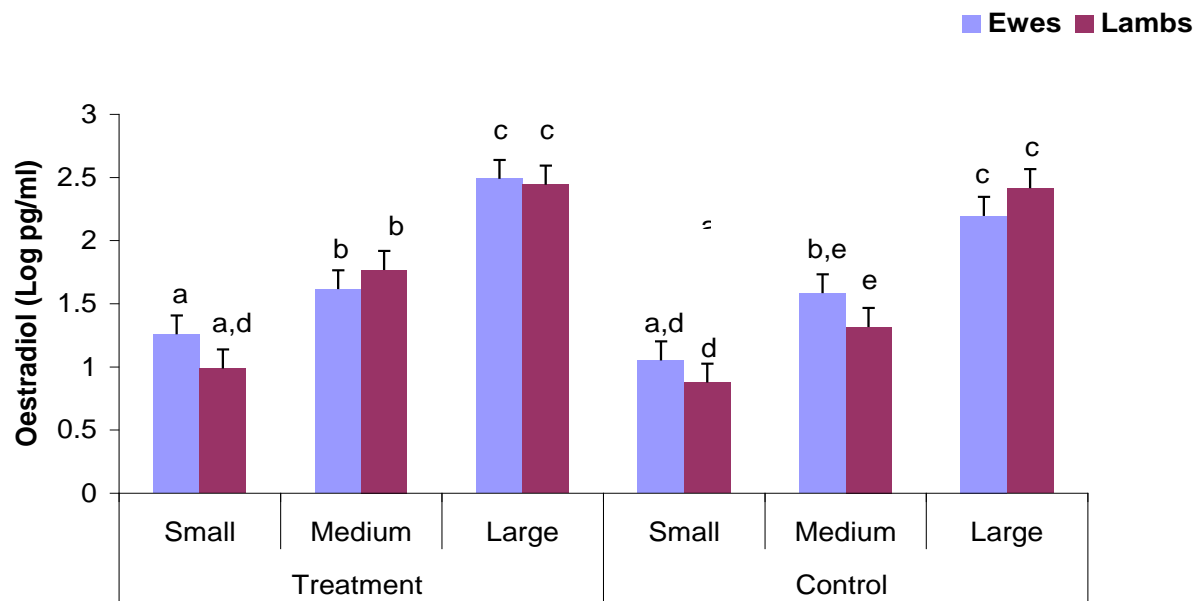


Figure.4.1 Effect of gonadotrophin on age and follicle size on the mean oestradiol concentrations in ewes and ewe lambs half follicles media cultured in TCM 199, with or without FSH/LH (SED = 0.064). Bars with different letters are significantly different (n = 20 follicles for each size/treatment per age).

4.1.1.2 Progesterone

Over all progesterone concentrations were significantly higher ($P < 0.05$) in media from ewe lambs follicles than those from ewes, when cultured with or without gonadotrophin and incubated for 24 h (Mean of log progesterone in ewes follicles media was 0.887 ng/ml and in ewe lambs media was 1.157 ng/ml, SED = 0.023). Furthermore, a similar result was found between ewe follicles of different size ($P < 0.05$), as small follicles produced less progesterone

than medium, and medium produced less than large follicle size. However, in ewe lambs, progesterone produced from follicles of different size was similar (Figure.4.2).

Error! Not a valid link.

Figure.4.2. Effect of follicle size on the mean progesterone concentrations in ewes and lambs half follicles media were cultured 24 h with or without FSH/LH (SED = 0.057). Bars with different letters are significantly different ($P < 0.05$), ($n = 12$ follicles for each size/treatment per age).

4. 3. 1. 2. Follicles cultured in TCM-199 with different ratios of FSH/LH for different incubation times

4. 3. 1. 2.1 Oestradiol concentrations in media from follicles cultured with FSH/LH

Overall oestradiol concentrations were significantly ($P < 0.05$) greater in media from ewes follicles than media from ewe lambs follicles (mean log oestradiol concentration of ewes was 2.937 pg/mg and for ewe lamb was 2.761 pg/mg (SED = 0.05). No difference was found with time of incubation in either ewes or ewe lambs, but oestradiol concentration in ewes follicle media was significantly higher ($P < 0.05$) than in media of ewe lambs follicles when cultured for 4 and 6 h (Figure.4.3). Moreover, oestradiol concentrations tended to decrease with increasing time of incubation in both age of groups (Figure.4.3 and Appendix.4.2). Whereas no difference was found between ewes and ewe lambs with different dilutions of FSH/LH in overall times of incubation (Appendix.4.3A, B, C and D).

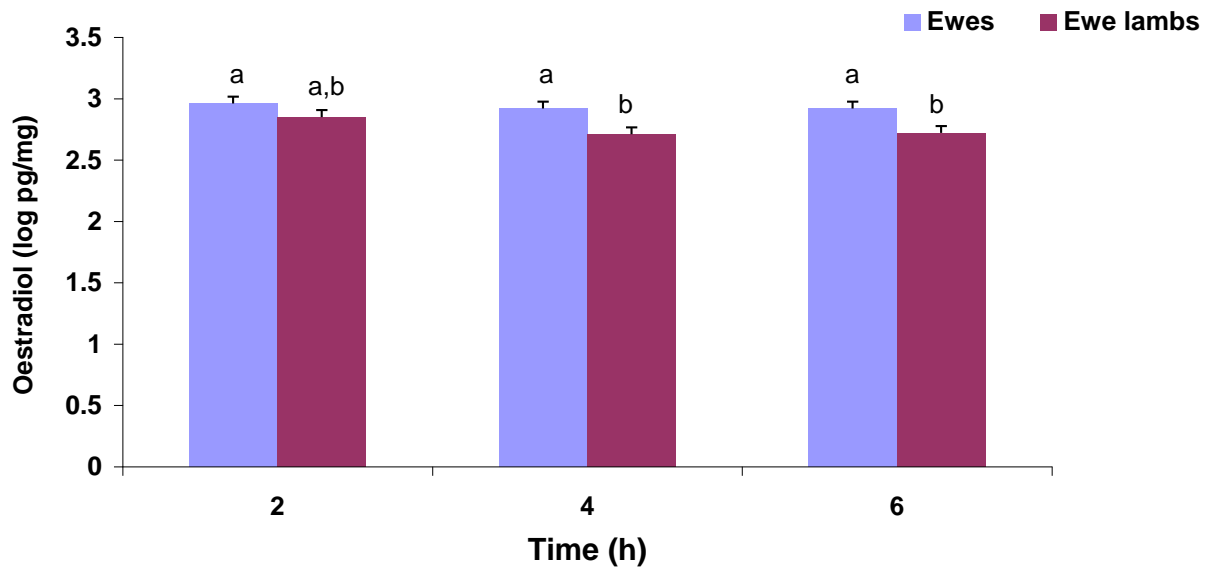


Figure.4.3. Effect of animal age on the mean oestradiol concentrations in ewes and ewe lambs ¼ follicles cultured in different concentrations of FSH/LH (SED = 0.056). Bars with different letters are significantly different ($P < 0.05$) ($n=18$ follicles for each time per age).

4.3.1.2.2 Progesterone concentrations in media from follicles cultured with FSH/LH

The results showed no significant difference ($P > 0.05$) in progesterone concentration between ewe and ewes lambs media when follicles cultured with different concentrations of FSH/LH hormones. Progesterone concentrations were higher ($P < 0.05$) at 0.5/5 and 0/0 ng/ml than at 0.005/0.05 and 0.05/0.5 ng/ml (Appendix.4.4). Furthermore, ewes follicles produced significantly ($P < 0.05$) more progesterone at 0/0 than at 0.005/0.05 and at 0.5/5 ng/ml, (Figure.4.4), but no differences were found between the other dilutions. Ewe follicles produced significantly ($P < 0.05$) more progesterone than ewe lamb follicles at 0.05/0.5 ng/ml.

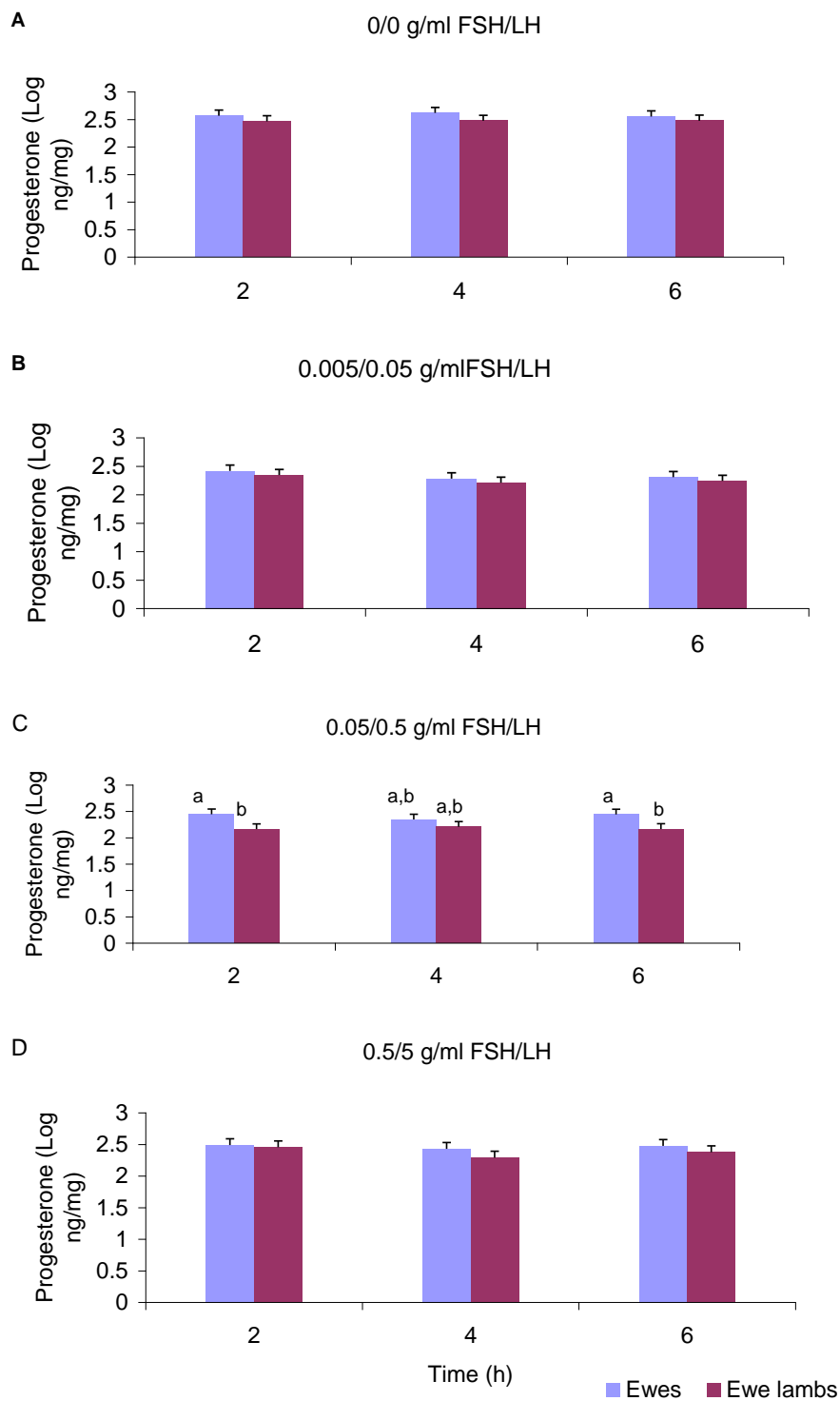


Figure.4.4 Effect of gonadotrophin hormones and age on the mean progesterone concentrations in ewes and ewe lambs $\frac{1}{4}$ follicles cultured in FSH/LH (SED = 0.01). Bars with different letters are significantly different ($P < 0.05$) ($n = 72$ follicles per age).

4.3.1.2.3 Oestradiol concentrations in media of follicles cultured with different concentrations of hCG

Overall oestradiol levels showed a significant difference ($P < 0.05$) between ewes and ewe lambs in media from follicles, mean of log oestradiol in ewes follicles media was 2.8 pg/mg and in ewe lambs follicles media was 2.6 pg/mg, (SED = 0.046). Moreover, oestradiol concentrations gradually increased (Figure.4.5) with increasing time of incubation, but the increase was not significant. In addition no difference was observed in oestradiol levels among different dilutions of hCG, or among the interaction between time and age, or between dilution, time and age ($P > 0.05$) (Figure.4.5).

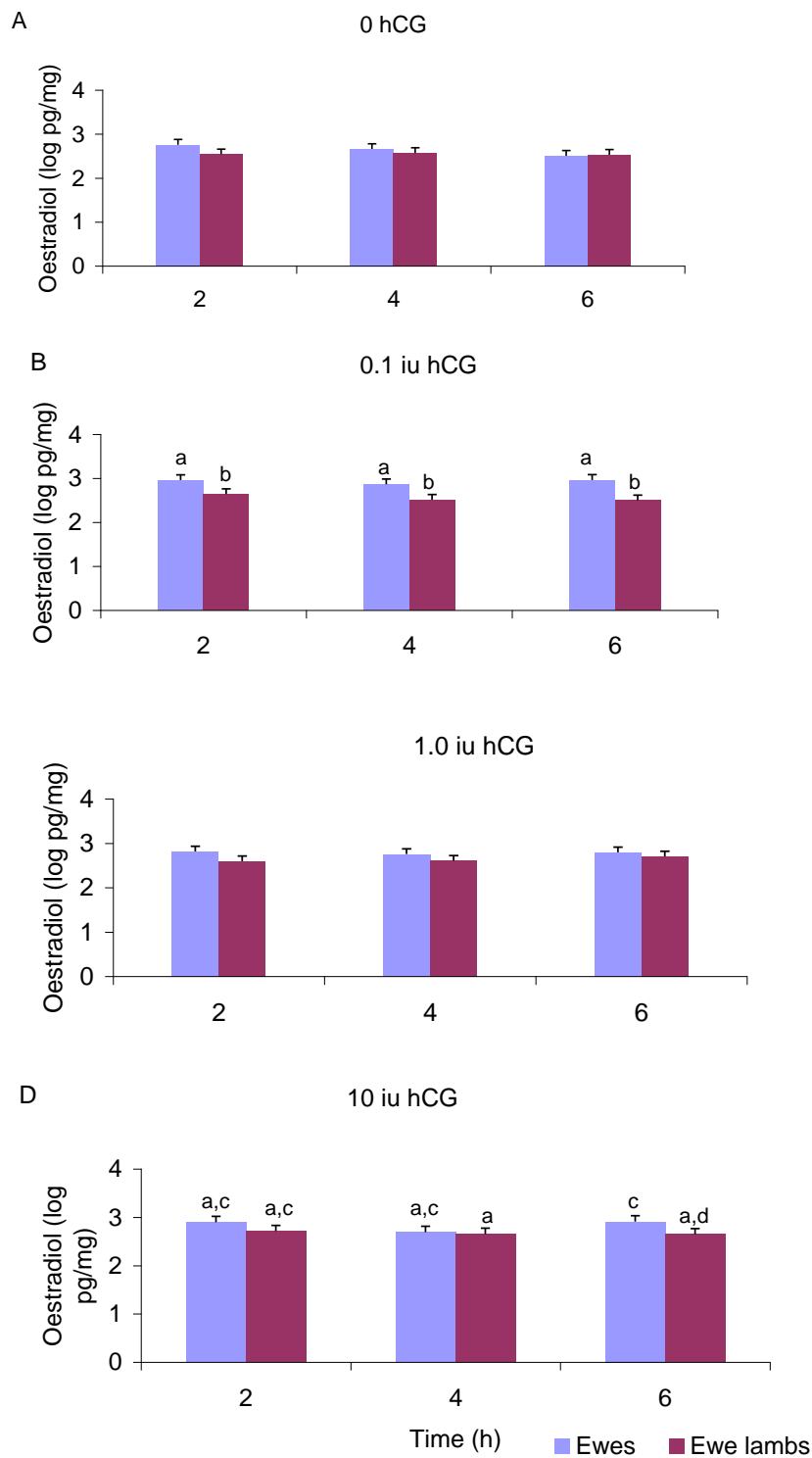


Figure.4.5 Effect of hCG dilutions on the mean of oestradiol concentrations in ewes and ewe lambs ¼ follicles media (SED = 0.12). Bars with different letters are significantly different ($P < 0.05$) ($n = 72$ follicles per age).

4. 3. 1. 2.3 Progesterone in media of follicles cultured with different concentrations of hCG

The results from this assay showed that a significant difference ($P < 0.05$) was found between ewes and ewe lambs progesterone concentrations in the media from follicles cultured in different concentrations of hCG, mean log progesterone in ewe follicles media was 2.66 ng/mg and for ewe lamb was 2.46 ng/mg (SED = 0.035). Furthermore, there was a significant difference ($P < 0.05$) among hCG dilutions, 1.0 and 10.0 iu/ml produced more progesterone than 0.1 iu/ml in ewe lamb but not in ewe follicles media. However, no difference was found between the other dilutions in both ages of groups (Figure.4.6 and Appendix.4.5).

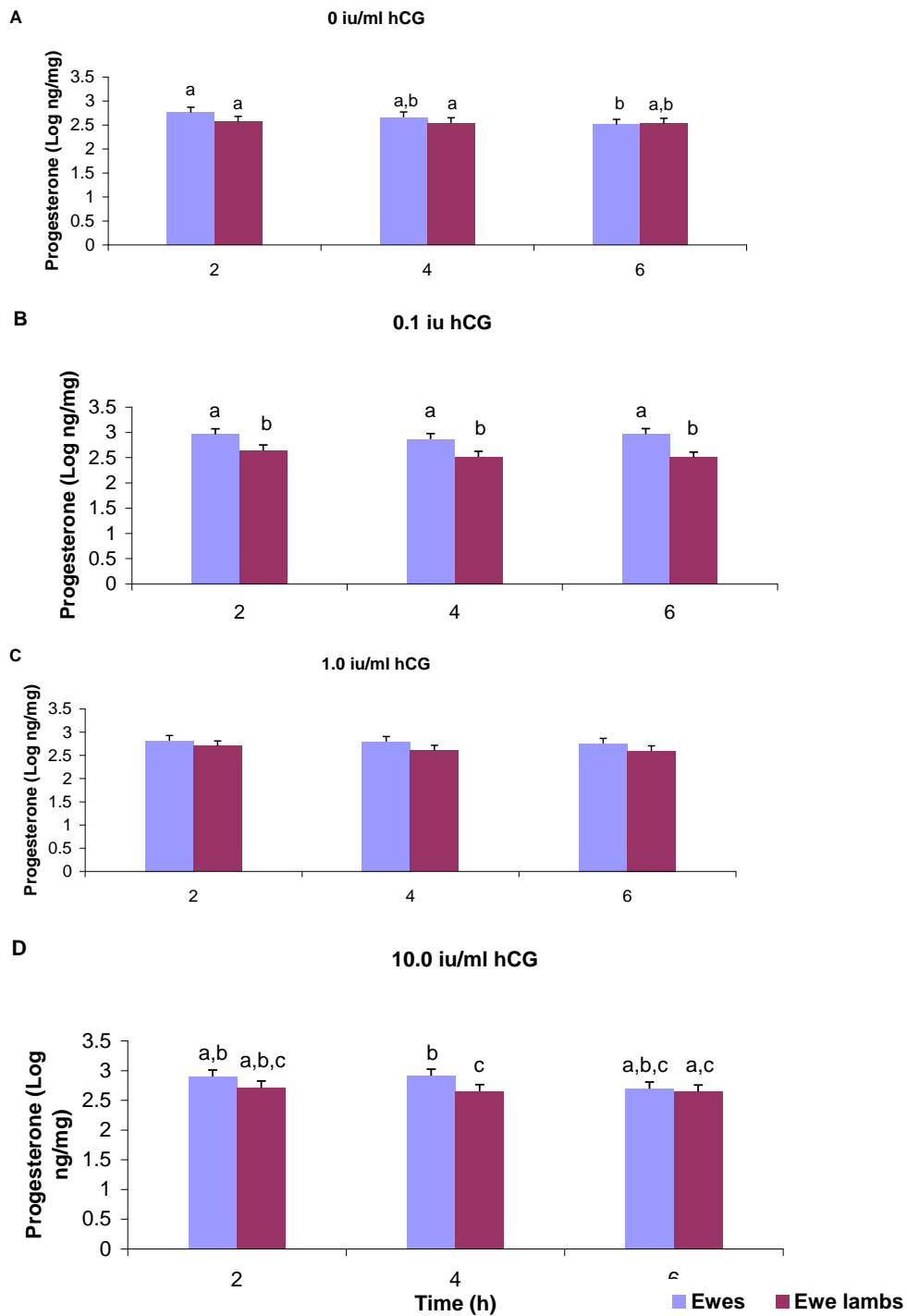


Figure.4.6 Effect of hCG concentrations on the mean progesterone concentrations in ewes and ewe lambs ¼ follicles cultured in different amounts of hCG (SED = 0.054). Bars with different letters are significantly different (n = 72 follicles per age).

4.3.2 CL cultured in LH

4.3.2.1 Progesterone concentrations in media from CL culture with four different concentrations of LH for 24 hours

Ewe CLs significantly ($P < 0.05$) produced more progesterone than ewe lamb CL when cultured for 24 h with different amounts of LH (Mean log progesterone concentration in ewe CL media was 1.62 ng/mg and for ewe lamb was 1.40 ng/mg). However, there was no difference ($P > 0.05$) found in the interaction between age and dilution (Appendix.4.5). Moreover, no difference was found between the different dilutions of LH in progesterone concentrations (Mean log progesterone was 1.476, 1.512, 1.533 and 1.51 μ g/mg for 0, 10, 100 and 1000 ng/ml LH respectively, SED = 0.04).

4.3.2.2 Progesterone concentration in the tissue of CL cultured in different concentrations of LH for 24 hours.

In overall there were no differences ($P > 0.05$) between both ewes and ewe lambs in progesterone secreted from cultured CL, mean log progesterone in ewe CL was 1.561 ng/mg and 1.56 ng/mg in ewe lamb, (SED = 0.057). In addition no differences observed on the interaction between age and dilution ($P > 0.05$). A significant difference ($P < 0.05$) was observed in tissue progesterone concentration for both ewe and ewe lamb CL cultured with different LH incubation. For example, 1000 ng/ml produced more progesterone than 0.0 and 10 ng/ml, whereas no difference found between 0.0 and 10 ng/ml or between 100 and 1000 ng/ml (Figure.4.7). There was no difference found between the two ages, nor in the age with dilution interaction (Appendix.4.6).

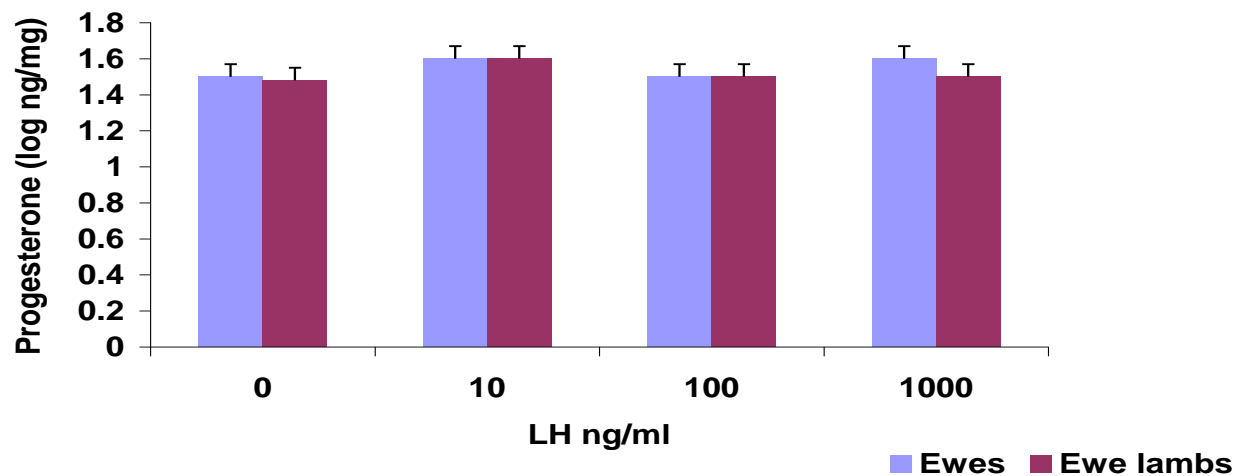


Figure.4.7 Effect of LH dilution on the mean progesterone secreted from ewes and ewe lambs cultured CL (SED = 0.07) and n=30 CL for each dilution per age.

4.3.2.3 CL cultured in four different concentrations of LH and cultured for 2, 4 and 6 hours.

Overall, ewe lambs' cultured tissue secreted significantly ($P < 0.05$) more progesterone than ewes tissue during all incubation times (Figure.4.8). The results showed that ewe lambs CL produced significantly ($P < 0.05$) more progesterone than ewes CL tissue when cultured in different amounts of LH during different times of incubation. Mean log progesterone in ewe CL was 0.23 ng/mg and for ewe lambs was 0.31 ng/mg, (SED = 0.02). Overall in both ewes and ewe lambs cultured CL progesterone concentration decreased with increasing of time incubation (Appendix.4.7).

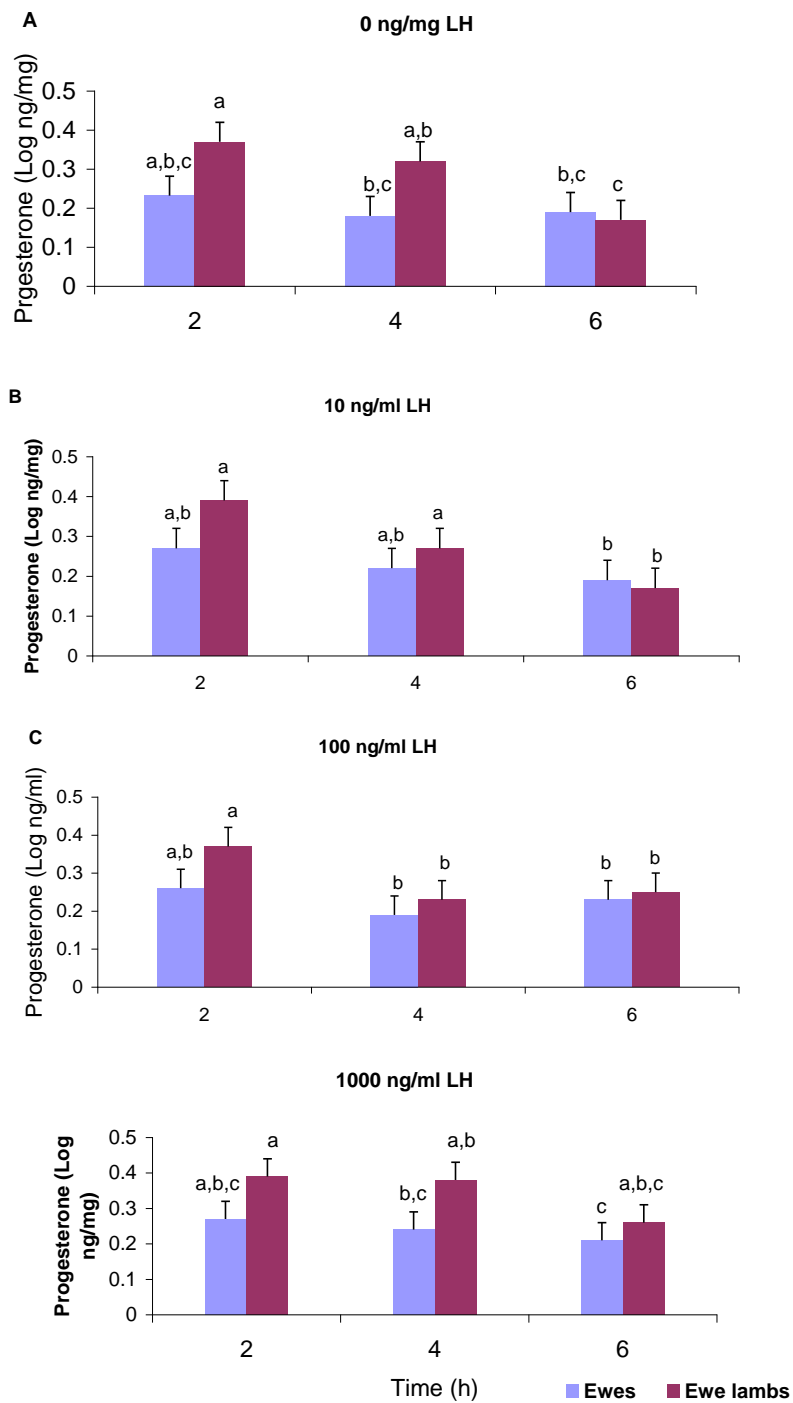


Figure.4.8 Effect of incubation time on the mean progesterone concentrations in ewes and ewe lambs CL cultured media, in different amounts of LH, (SED = 0.05). Bars with different letters are significantly different, (n = 60 CL pieces for ea/timeper age).

4.3.3 CL cultured in four different concentrations of hCG for 2 and 4 hours.

In overall ewes CLs produced significantly more ($P<0.05$) progesterone than ewe lambs CL after 2 h incubation (Mean log progesterone ewes CL was 0.347 ng/mg and in ewe lambs CL was 0.30 ng/mg, SED = 0.023), but CLs of ewe lambs showed no significant difference in media levels of progesterone produced compare to ewes after 4 h of incubation, mean log progesterone from ewes CL was 0.21 ng/mg and from ewe lambs was 0.25 ng/mg, (SED = 0.023). Furthermore, in both age groups of animal, CL tissues produced significantly more ($P<0.05$) progesterone after 2 h than after 4 h of incubations (Appendix.4.8). In both groups, CL tissue produced significantly ($P<0.05$) more progesterone without hormone (0.0) than after 0.1 or 1.0 and 10.0 iu/ml (Figure.4.9)

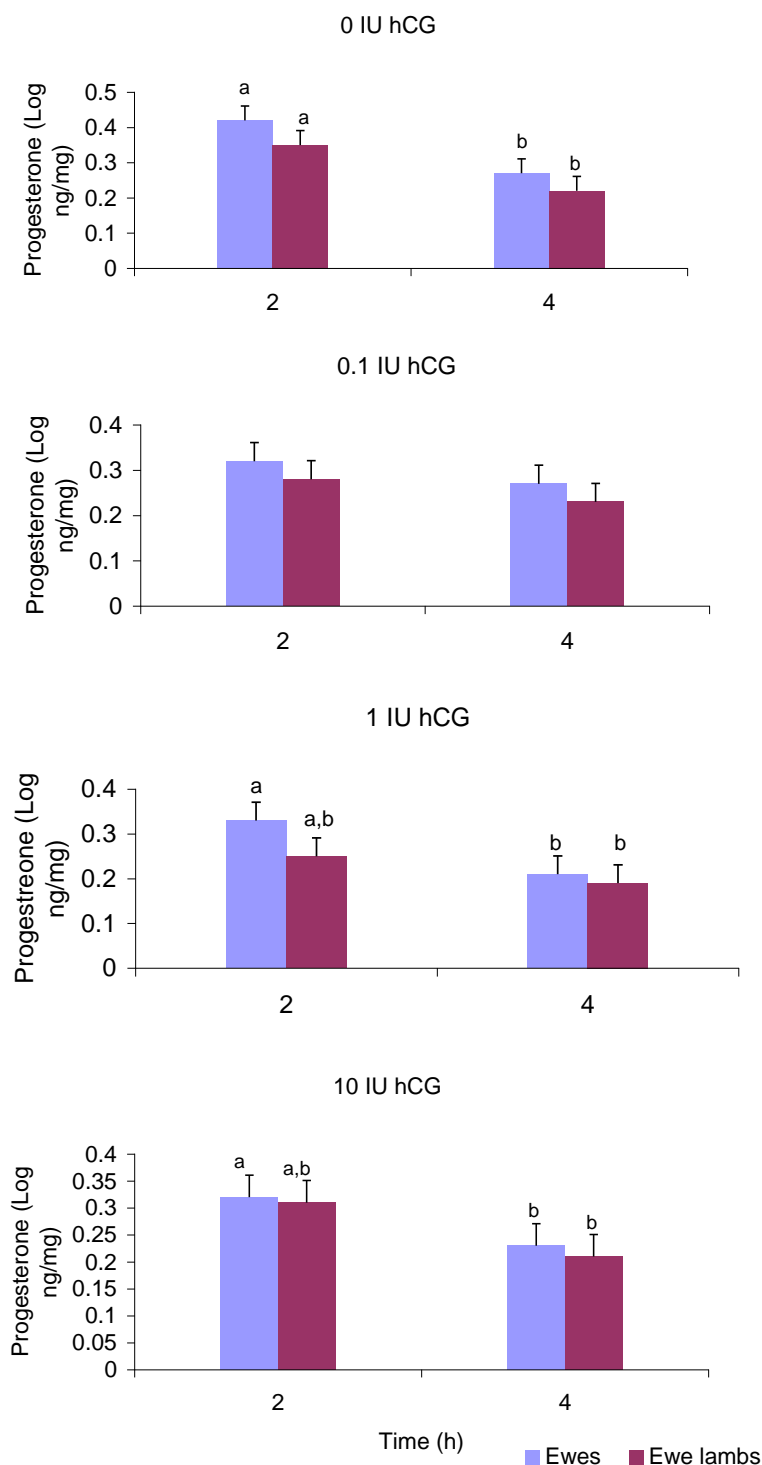


Figure.4.9 Effect of hCG concentrations on the mean progesterone in ewes and ewe lambs CL media cultured in TCM199 with different amount of hCG (SED = 0.041). Bars with different letters are significantly different (n = 48 CL pieces/time per age).

4.3.4 Proteomics of small medium and large follicles from ewe lambs and ewes incubated with FSH and LH

4.3.4.1 Ewes small follicular shells

Most abundance spots estimated at 20.1 to 97 kDa molecular weight (MW) and (46%) matching percent where control gels were used as a standard gel for all proteomics gels in this chapter. The unique spots in treatment gel were estimated at 20.1 to 66 kDa marker and ranging between *pI* 6-8 (Figure.4.10 A and B). Two protein spots were chosen as example for up and down regulated. Spot 750 which estimated at 97 kDa marker and *pI* 6-8 showed an increase 2.7-fold and spot 832 showed a decrease 2.45-fold and estimated at 97 kDa marker and *pI* 7 to 8 as shown in Figure.4.11 A and B and Figure.4.12.

Table.4.1 Treatment with FSH/LH and control ewe gels from small follicular shells.

Spots	Ewes LH/FSH	Ewes control
Total number of spots	207	131
Matched %	46	100
Up regulated 2 fold	34	11
Down regulated 2 fold	11	34
Between \pm 2 fold	53	51
Unique spots	109	35

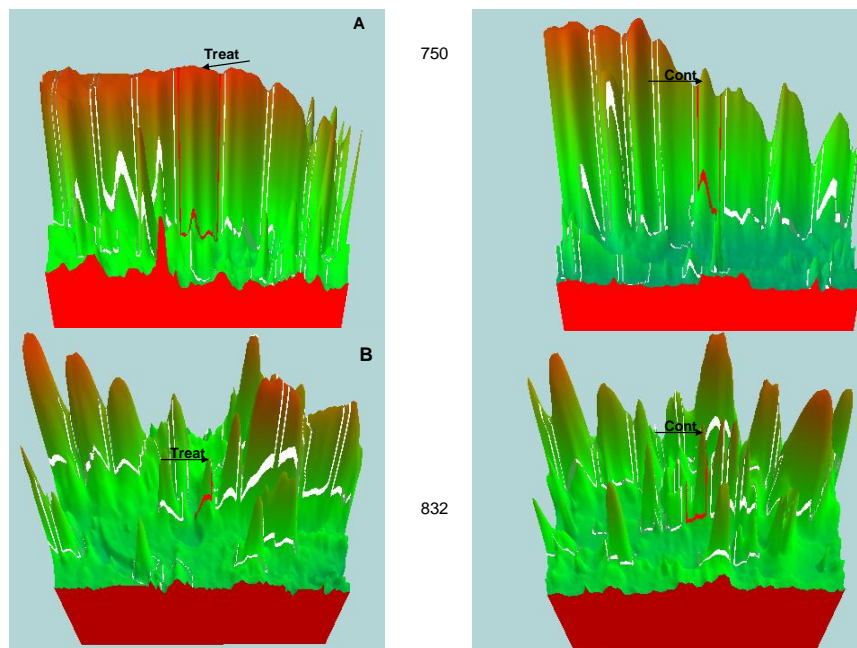


Figure.4.11 Three dimension image of spot 750 up-regulated 2.7-fold gel (A) and spot 832 down regulated 2.45-fold (B) in treatment and control in ewe small follicular shells gels.

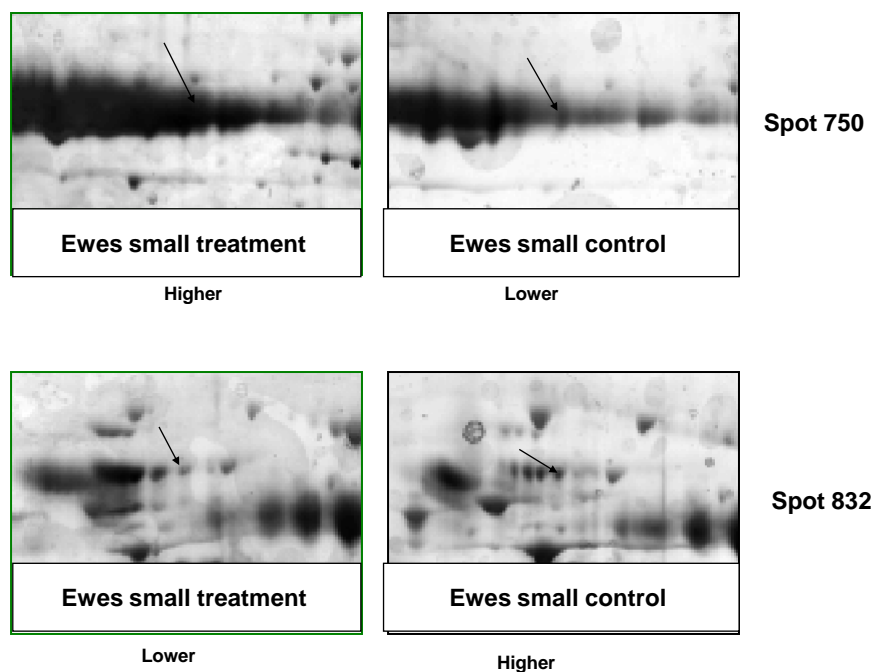


Figure.4.12 Montage images for up and down regulated spots of treatment and control small ewe follicular shells.

4.3.4.2 Ewes medium follicular shells

Data from ewe medium follicular shells gels showed more spots than small gel and less protein spots than in large follicular gels. Moreover, the same findings between treatment and control were found where the treatment gel contains less spots than control (Table.4.2) with a good matching percent (79%). The abundance protein spots estimated at 14.4 to 100 kDa and ranging between *pI* 5-7 (Figure.4.13 A and B). Two spots were taken as example for up and down regulated, spot 1910 estimated at 10-14.4 kDa and *pI* 5-6 showed increase 7.38-fold and spot, 1678 at 30.0-45.0, showed decrease 3.59-fold (Figure.4.14 A and B and Figure.4.15).

Table.4.2 Treatment with FSH/LH and control ewe gels from medium follicular shells.

Spots	Ewes LH/FSH	Ewes control
Total number of spots	269	345
Matched %	79	100
Up regulated 2 fold	21	61
Down regulated 2 fold	61	25
Between \pm 2 fold	133	131
Unique spots	54	128

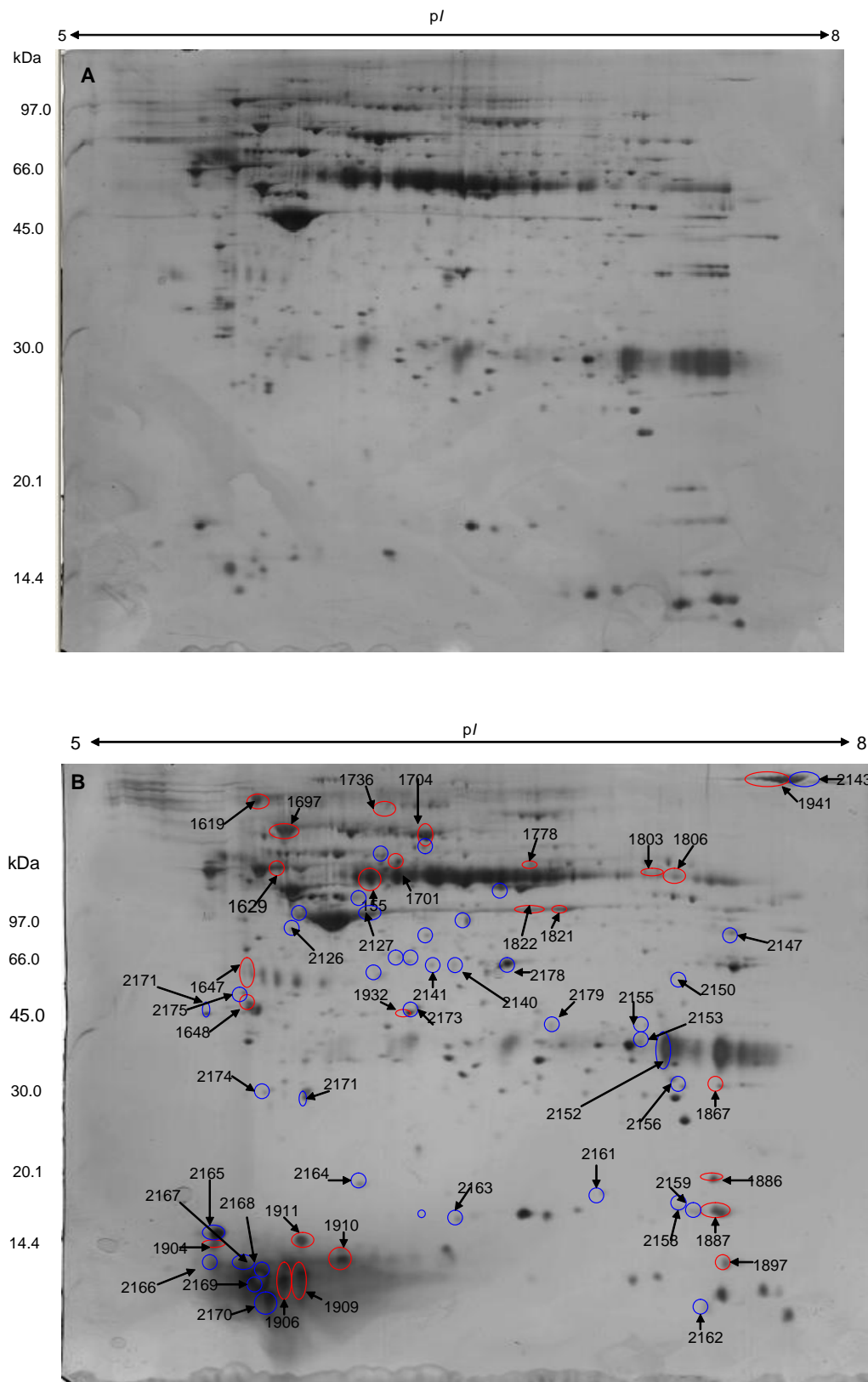


Figure.4.13 Pattern of protein secreted by ewe medium control treated follicular shells (A), and from medium follicles cultured with FSH/LH (B), showing up-regulated 2-fold spots (red circle) and unique spots (blue), and spots without circle are less than 2-fold.

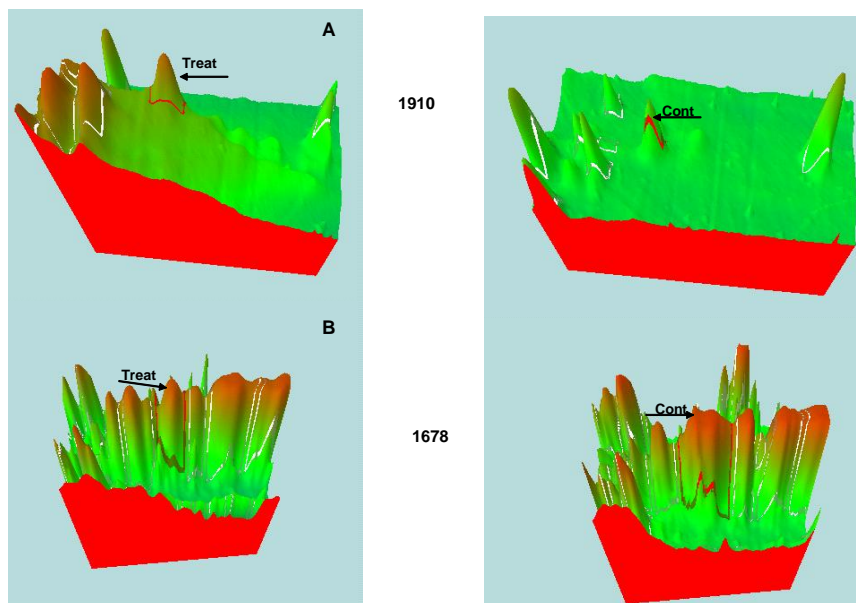


Figure.4.14. Three dimension image of spot 1910 up-regulated 7.38-fold gel (**A**) and spot 1678 down regulated 2.45-fold (**B**) in treatment and control in ewe medium follicular shells gels.

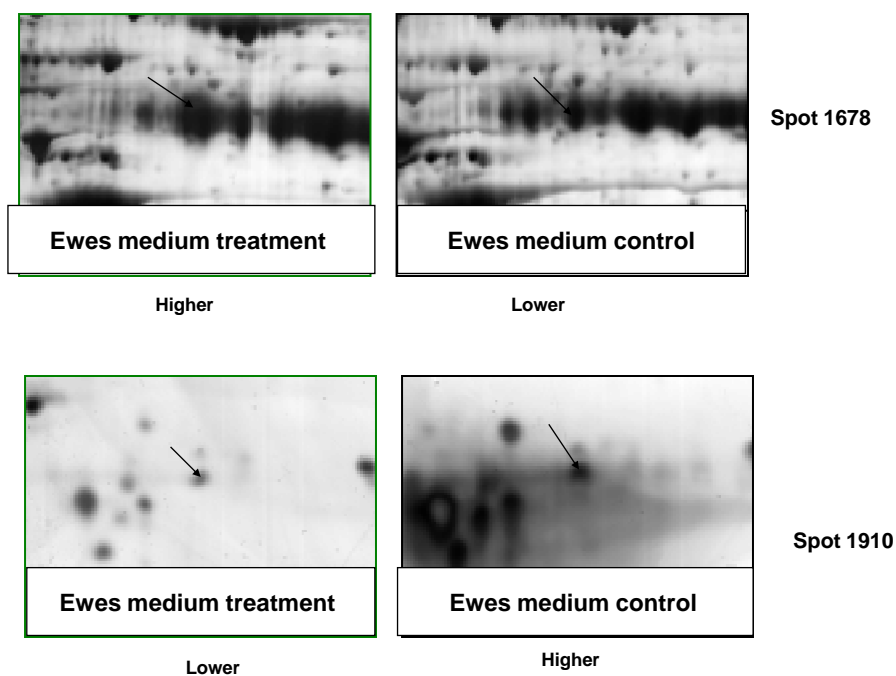


Figure.4.15 Montage images for up and down regulated spots of treatment and control medium ewe follicular shells.

4.3.4.3 Ewes large follicular shells

The analysis data from both treated and control shells gels appeared to contain a large proportion of protein spots, with treated gels containing more spots comparing with control gel, still percent recorded a good of matching spots (69%). The information for these spots is shown in (Table.4.3). Both gels appeared to contain large proportion of proteins of high MW ranging from 30 to 100 kDa. The high MW spots seen initially, distributed between *pI* 5-7 (Figure.4.16 A and B). Two protein spots were taken as example, spot 1115 estimated at 10.0-14.4 kDa, showed a 2.2-fold increase and spot 1154 estimated at 66-100 kDa, showed 2.76-fold decrease (Figure.4.17 A and B and Figure.4.18). In addition dramatic increase in protein numbers was seen between the 20.1 and 97.0 kDa marker.

Table.4.3 Treatment with FSH/LH and control ewe gels from large follicular shells.

Spots	Ewes LH/FSH	Ewes control
Total number of spots	418	330
Matched %	69	100
Up regulated 2 fold	58	42
Down regulated 2 fold	42	58
Between \pm 2 fold	190	189
Unique spots	128	41

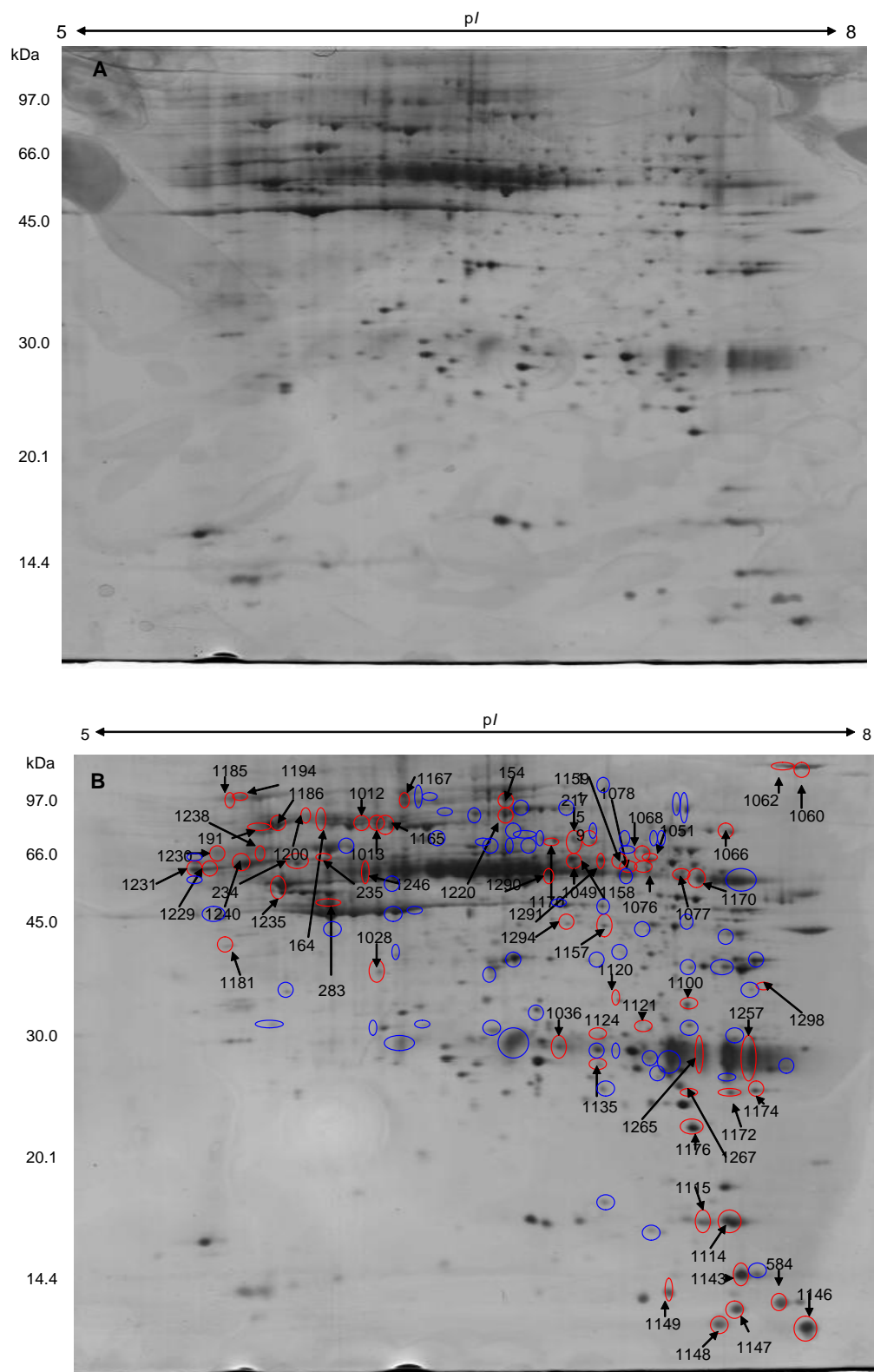


Figure.4.16 Pattern of protein secreted by ewe large control treated follicular shells (A), and from large follicles cultured with FSH/LH (B), showing up-regulated 2-fold spots (red circle) and unique spots (blue), and spots without circle are less than 2-fold.

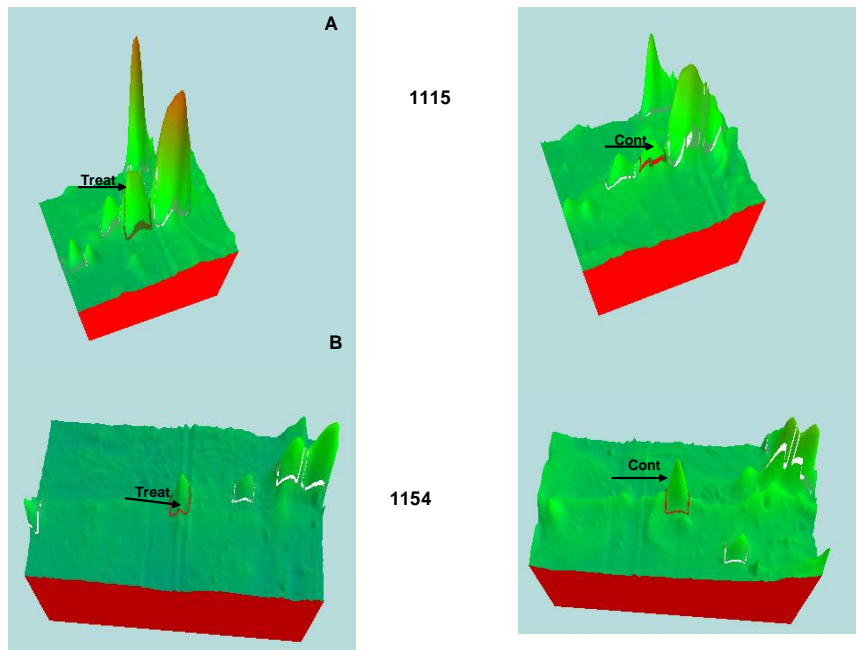


Figure.4.17 Three dimension image of spot 1115 up-regulated 2.2-fold gel (A) and spot 1143 down regulated 2.76-fold (B) in treatment and control in ewe large follicular shells gels.

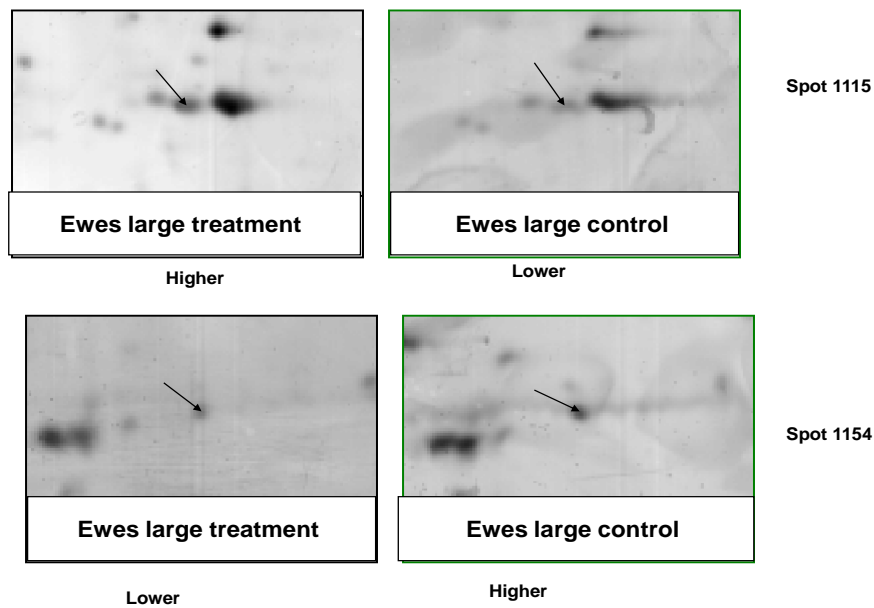


Figure.4.18 Montage images for up and down regulated spots of treatment and control large ewe follicular shells.

4.3.4.4 Ewe lambs small follicular shells

The results for the small follicle gels for ewe lamb showed less protein spots compared with the small follicle ewe gels and small follicle ewe lamb control gels contains a greater number of spots compared to the treatment gel (Table.4.4). Overall small ewe lamb follicular shells gels contain less spots than medium gels, mainly, majority of spots estimated at 66 to 100 kDa marker. Moreover, the up regulated spots in treatment gel were estimated on the same position of marker (Figure.4.19 A and B) with good matching percent (68%). Moreover, the interested two spots were estimated at 66.0 to 100 kDa, and ranging between *pI* 5-7. Spot 526 increase a 3.03-fold and spot 572 decrease a 2.12 (Figure.4.20 A and B and Figure.4.21).

Table.4.4 Treatment with FSH/LH and control ewe lamb gels from small follicular shells.

.Spots	Lambs LH/FSH	Lambs control
Total number of spots	68	85
Matched %	68	100
Up regulated 2 fold	8	9
Down regulated 2 fold	9	8
Between \pm 2 fold	13	31
Unique spots	38	37

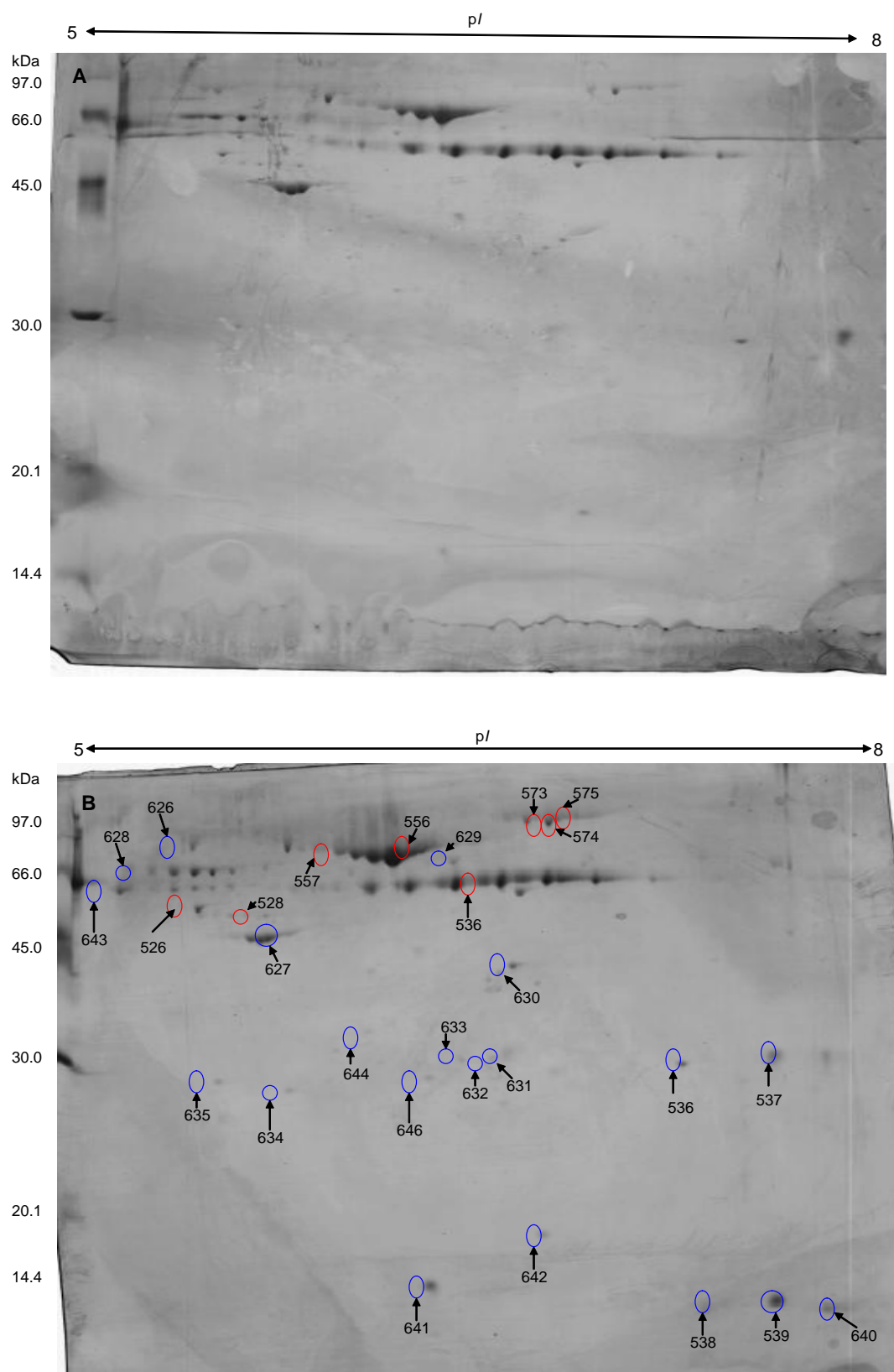


Figure.4.19 Pattern of protein secreted by ewe lamb small control treated follicular shells (**A**), and from small follicles cultured with FSH/LH (**B**), showing up-regulated 2-fold spots (red circle) and unique spots (blue), and spots without circle are less than 2-fold.

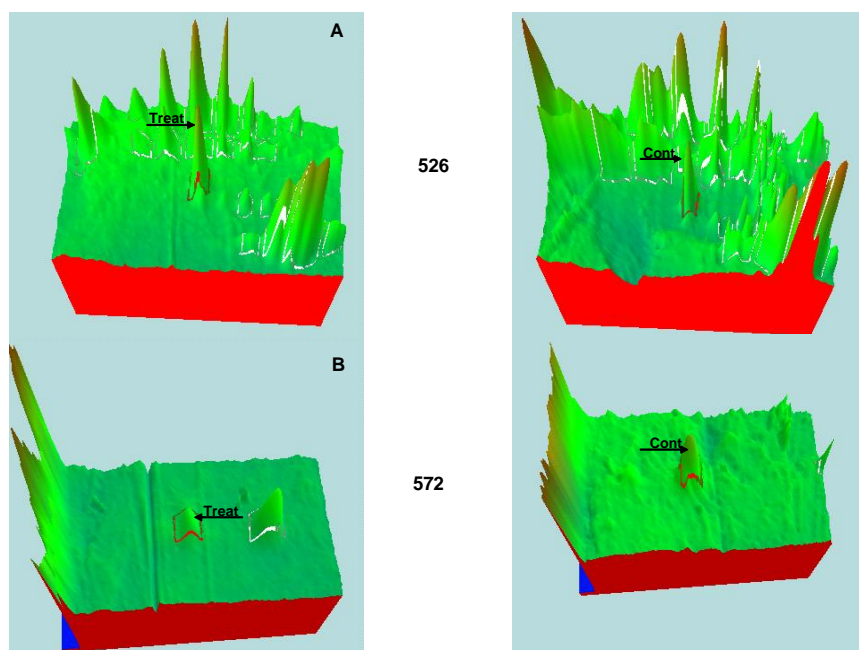


Figure.4.20 Three dimension image of spot 526 up-regulated 3.03-fold gel (A) and spot 572 down regulated 2.12-folds (B) in treatment and control in ewe lamb small follicular shells gels.

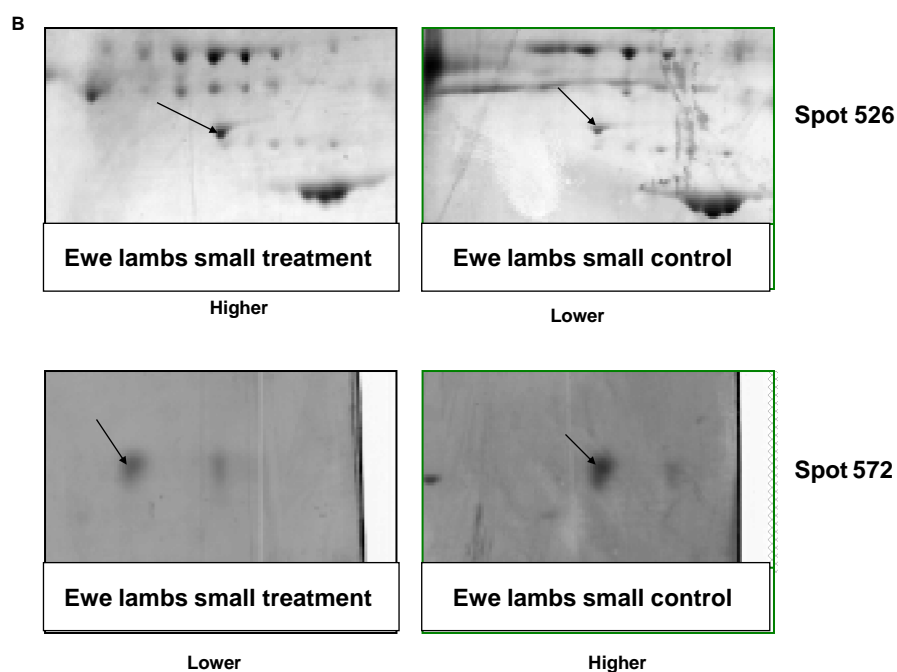


Figure.4.21 Montage images for up and down regulated spots of treatment and control small ewe lamb follicular shells.

4.3.4.5 Ewe lambs medium follicular shells

The results information from this experiment showed in (Table.4.5). Upregulated spots was less in treatment gel compare with control gel, and showed much reduced matching levels with only 32%. However, the down regulated and unique spots were more in treatment gel than in control. In addition the up regulated protein spots in treatment gel estimated at 66.0-97.0 and *pI* 5-7 (Figure.4.22 A and B). Two spots were shown to give an example for the up regulated 488 estimated at 97 kDa, and *pI* 5-7 (increase 2.62-fold) and down regulated spot 667 (decrease 4.43-fold) and estimated at 66-100 kDa marker and raining between *pI* 5-7 (Figure.4.23 A and B and Figure.4.24). The abundance protein unique spots in the treatment gel were distributed at 10.0 to 30.0 kDa and *pI* 6-8.

Table.4.5 Treatment with FSH/LH and control ewe lamb gels from medium follicular shells

Spots	Lambs LH/FSH	Lambs control
Total number of spots	125	93
Matched %	32	100
Up regulated 2 fold	11	17
Down regulated 2 fold	17	11
Between \pm 2 fold	43	12
Unique spots	54	53

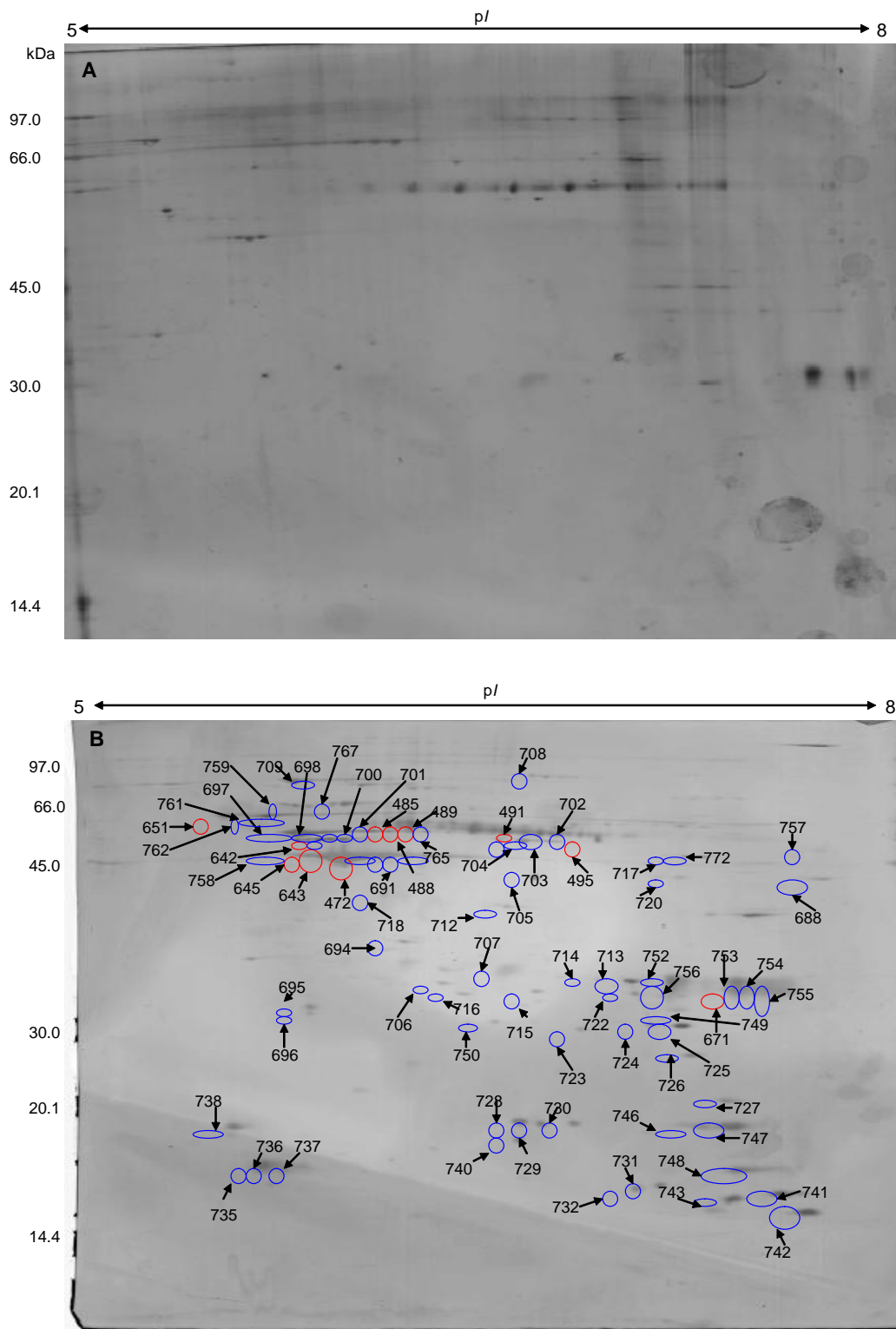


Figure.4.22 Pattern of protein secreted by ewe lamb small control treated follicular shells (A), and from medium follicles cultured with FSH/LH (B), showing up-regulated 2-fold spots (red circle) and unique spots (blue), and spots without circle are less than 2-fold.

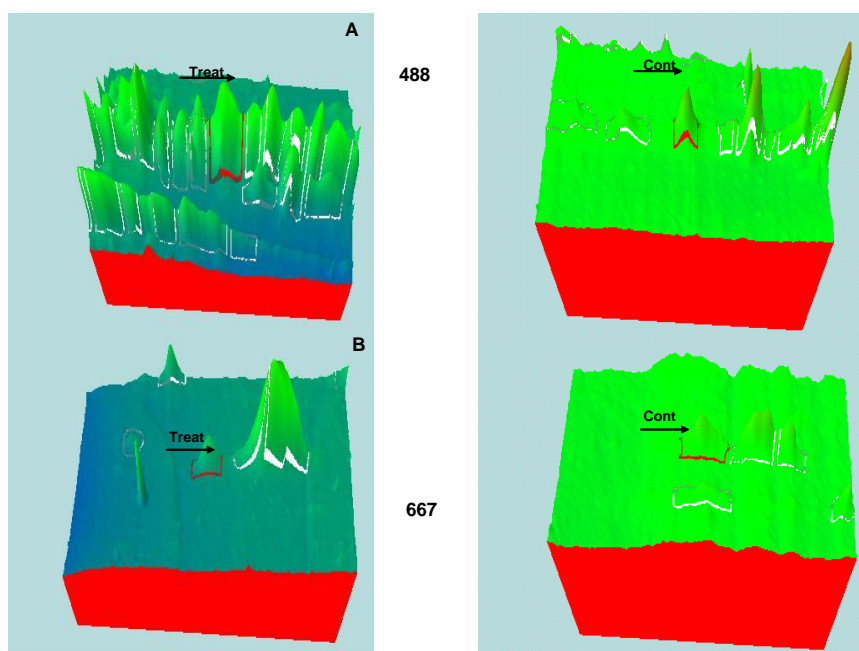


Figure.4.23 Three dimension of spot 488 up-regulated 2.62-fold gel (A) and spot 667 down regulated 4.43-folds (B) in treatment and control in ewe lamb medium follicular shells gels.

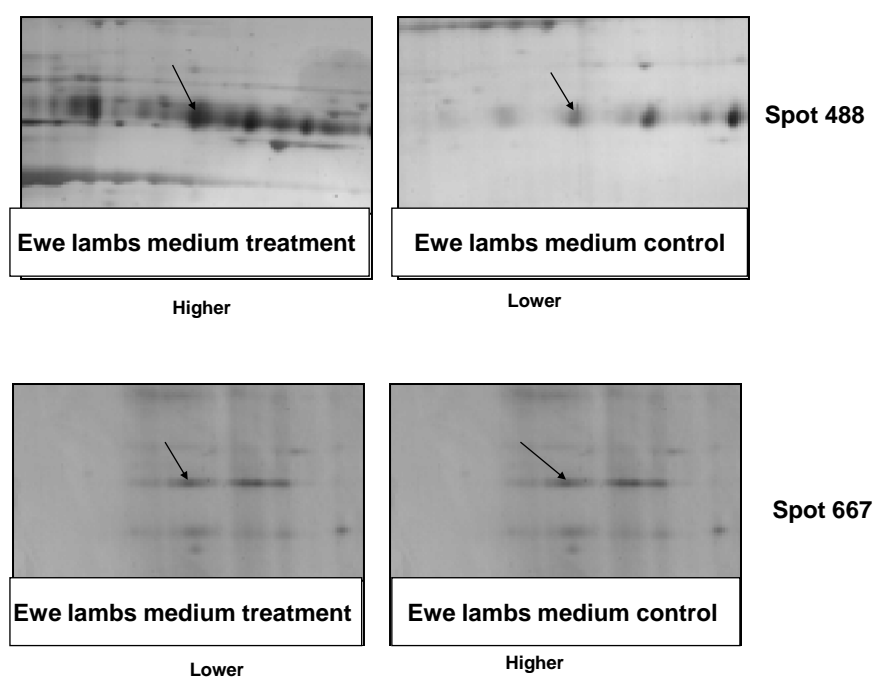


Figure.4.24 Montage images for up and down regulated spots of treatment and control medium ewe lamb follicular shells.

4.3.4.6 Ewe lambs large follicular shells

The data from this experiment revealed to increase the protein spots compared with medium gels spots (Table.4.6). Moreover, in both ewes and ewe lambs the protein spots number were increased with the increasing of follicle size, the protein profile in this experiment was similar to that found between treatment and control gels in both groups of animals. In treatment gel, most of the up regulated spots and unique spots are estimated at 30 to 100 kDa marker, moreover, the treatment gel contain more unique spots than control gel (Figure.4.25 A and B). The two interested spots are, spot 1168 estimated at 97 to100 kDa marker and ph 5-7, showed an increase a 2.23-fold and spot 1235 estimated at 45 to 100 kDa. And ranging between pI 6-8 showed decreased a 4.45-fold (Figure.4.26 A and B and Figure.4.27). Where the matching present was acceptable (63%) and most spots in treatment gel were unique spots then spots between < 2 fold and > 2 fold.

Table.4.6 Treatment with FSH/LH and control ewe lamb gels from large follicular shells.

Spots	Lambs LH/FSH	Lambs control
Total number of spots	253	220
Matched %	63	100
Up regulated 2 fold	41	44
Down regulated 2 fold	44	42
Between \pm 2 fold	76	75
Unique spots	92	59

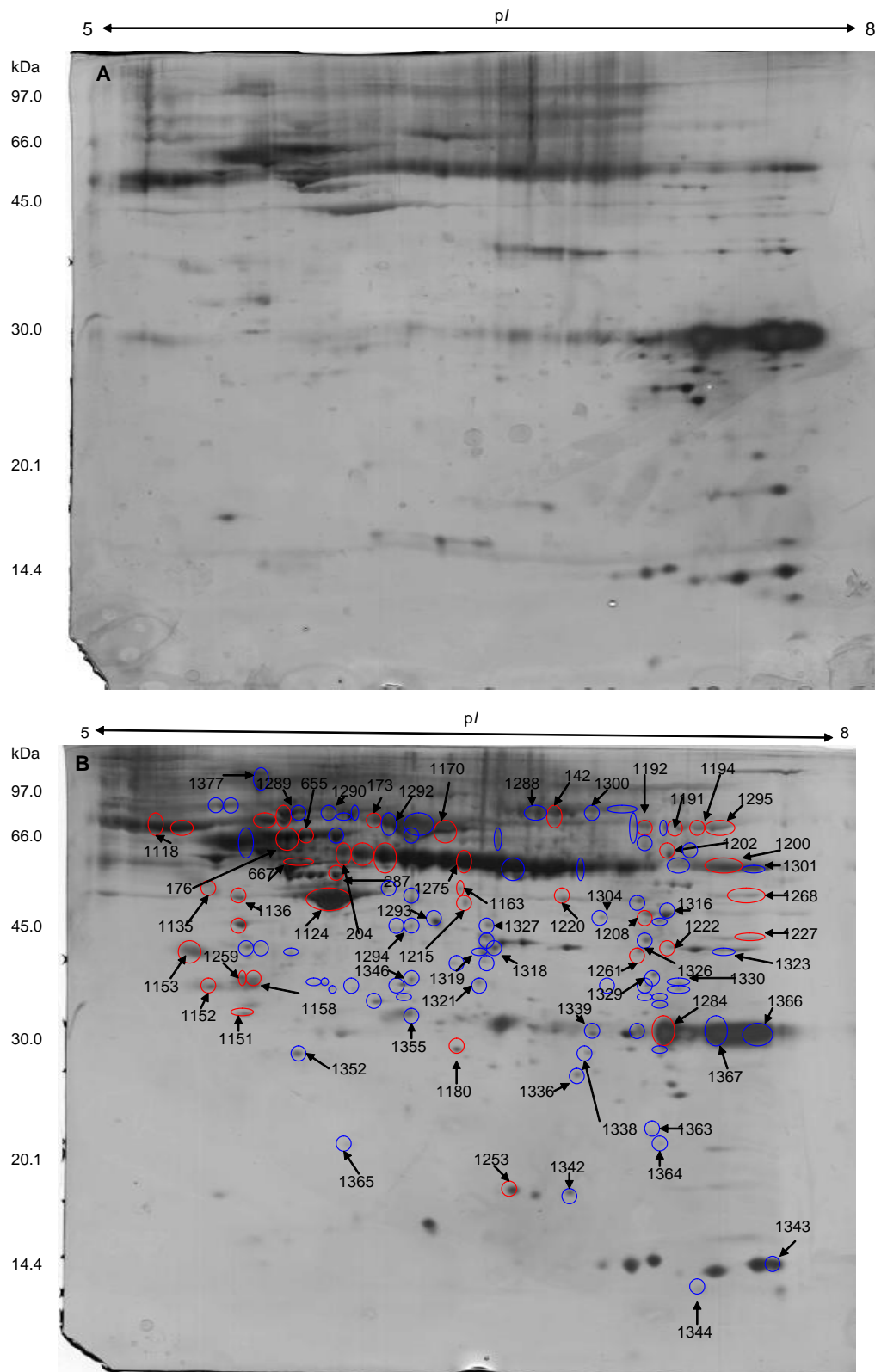


Figure.4.25 Pattern of protein secreted by ewe lamb large control treated follicular shells (A), and from large follicles cultured with FSH/LH (B), showing up-regulated 2-fold spots (red) and unique spots (blue), and spots without circle are less than 2-fold.

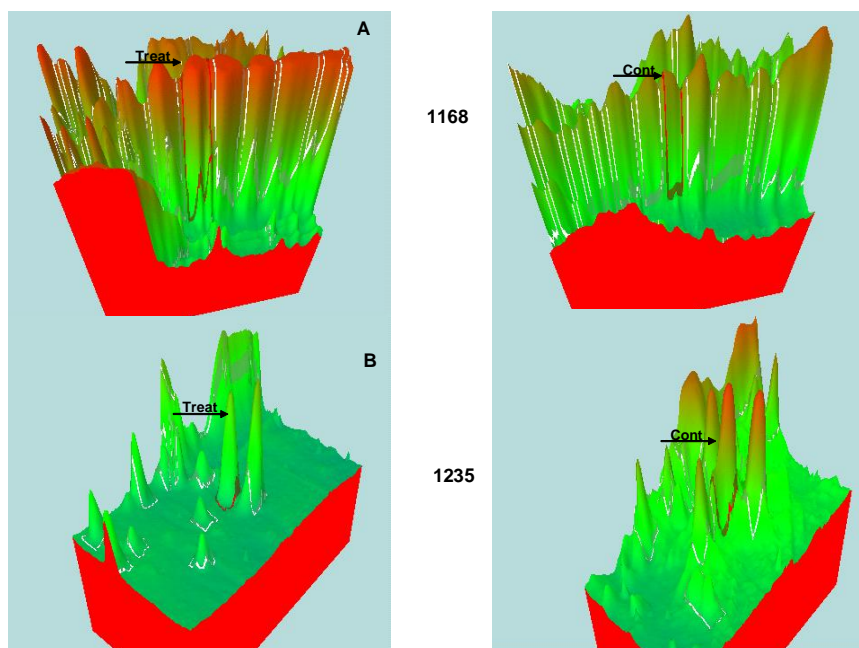


Figure.4.26 Three dimension image of spot 1168 up-regulated 2.23-fold gel (A) and spot 1235 down regulated 4.45-folds (B) in treatment and control in ewe lamb large follicular shells gels.

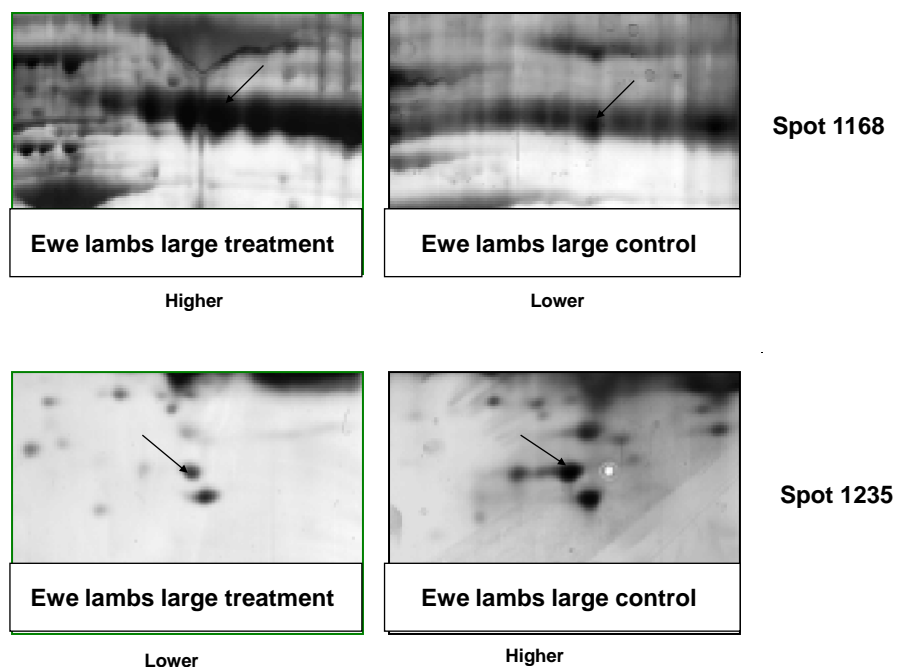


Figure.4.27 Montage images for up and down regulated spots of treatment and control large ewe lamb follicular shells.

In general, some variation was seen in the volume of the same spots in treatment and control gels in all follicular shells size for both ewes and ewe lambs gels. The most noticeable difference between ewes and ewe lambs proteomics was the increase in total spots numbers in each ewes gel size compared with gels of from the same size of ewe lambs. The full results tables for all proteomics experiments for this chapter shown in the CD (appendix.4).

4.4 Discussion

Oestradiol concentration was higher in media with gonadotrophin present than without gonadotrophin. This difference could be the result of different treatment responses. The treatment with LH and FSH stimulated theca and granulosa cells to conversion of cholesterol to oestradiol. Moreover, *in vivo* FSH plays a major role in the control of the development of ovulatory follicles, which leads to an increased level of oestradiol (Campbell *et al.*, 1995a, Valasi *et al.*, 2007). In addition the results reveal that oestradiol concentration is increased with the increase in follicle size, which is similar to the result found in the previous chapter, when follicles were cultured in TCM-199 only (Figure.4.1). The increase in oestradiol concentration reflects the number of theca and granulosa cells and the response of these cells to the gonadotrophin hormone which contributes significantly to the variation in oestradiol production. These results are in agreement with the *in vivo* data collected from follicles sheep (Mann *et al.*, 1992, Moor *et al.*, 1973, Webb *et al.*, 1989), and in mares (Beck *et al.*, 2003). However these findings disagree with Tambe and Nandedkar (1993) who reported, that in sheep oestrogen levels did not increase in small follicles when treated with FSH or LH. If the same weight of cultured follicular tissue is used from both follicle sizes, the same amount of hormone should be found for medium and large follicles. Alternatively, the increase in

oestradiol concentrations found with follicular size is due to an increase in the number of FSH receptors on the granulosa cells and LH receptors on the theca layer.

Progesterone concentration was higher in ewe lamb follicles media compared with ewe follicles media. This suggests that in the ewe follicles theca layer, the conversion of cholesterol into oestradiol occurred more rapidly than in the ewe lamb, also the ewe lambs granulosa cells could be experiencing spontaneous luteinization (Tambe and Nandedkar, 1993). It could also be possible that granulosa cells have to be exposed to LH in order for luteinising to occur (Channing, 1970). In addition, ewe follicles may become atretic, resulting in a decreasing in sensitivity of granulosa cells to gonadotrophins and enzyme activity (Tilly *et al.*, 1992b). Furthermore, the increase in progesterone concentration coincides with an increase in follicle size. These results concur with Tambe and Nandedkar. (1993) who reported that, FSH and LH had no effect on oestrogen secretion from small follicles.

The results of oestradiol from follicles cultured for different times in TCM-199 with FSH and LH provide further evidence that ewe lambs follicles produced less oestradiol than ewes and this corresponds with the result found in the first experiment of the previous chapter. Similar results were reported in cows (Driancourt *et al.*, 2001a). Whilst time had no significant effect on oestradiol production in either age group, these results concur with earlier studies that reported hormone secretion from follicles in sheep was maximum after one hour of culture (Tambe and Nandedkar, 1993). The reason for this happening in this experiment might possibly be explained by the fact that tissue tended to undergo atresia as incubation time increased, which appears clear in Figure.4.3, as there was no difference in oestradiol concentration between ewes and ewe lambs after 2 h of incubation, but there was a clear

decrease after 4 and 6 h. These findings correspond to those of Tambe and Nandedkar (1993) who found that, steroid secretion decreased after 1 h in culture media.

Ewe follicles when cultured in hormone supplemented media produced more progesterone than ewe lamb follicles. The higher level of progesterone produced in ewe follicles compare to ewe lambs is due to a higher number of theca cells and consequentially more progesterone conversion from these cells and secreted in the media. For the higher progesterone in 0/0 and 0.5/5, could be explained as an experimental error, as these results are unexpected. In addition Campbell *et al.* (1995a) concluded that there are marked differences in follicle response to FSH according to follicle size, therefore more progesterone is assumed to be produced in ewe follicles.

The results of follicles cultured in TCM-199 showed that oestradiol secreted was higher in ewe follicles compare to ewe lamb (Figure.4.5). These results are in agreement with the results found in sheep by Khan *et al.* (2007). The recent data could reveal that the enzyme activity and the number of cells in the ewe granulosa cells were more active than those in ewe lambs, as the response of granulosa cells in ewe lamb tissue to the media are inadequate to secrete the same amount of hormone produced by ewes. The increase in oestradiol secretion in media simply reflects an increase in the number of theca cells. Other explanations could be that ewe follicles contained more hormone receptors compared with the ewe lambs, which allows more binding sites in the ewe follicle shells and secretes more oestradiol, therefore produces more oestradiol. Furthermore, Moore *et al* (Moor *et al.*, 1973) found, that in ewes about 25% of the cultured follicles secreted large amounts of oestradiol into the medium after as little as 5 minutes after

the administration of PMSG *in vivo*, when compared with 5% of follicles from untreated sheep. That could explain the high levels of oestradiol in ewe follicles compared with ewe lambs.

Progesterone concentration was higher in the media of ewe luteal tissue in contrast with ewe lamb tissue. This is confirmed in the data reported by Beck *et al.* (1994b) when ewes and ewe lambs were injected with GnRH. This showed that in ewes CL tissue produced more progesterone than ewe lambs tissue and this reveals that the function of large cells compared to small cells is to secrete more progesterone as a response to the LH. The ewe lambs granulosa cells failed to undergo luteinisation which can secrete progesterone as those in adult ewes.

The data of this experiment demonstrates no difference found between ewes and ewe lambs in luteal tissue culture with different amounts of LH. Overall ewes luteal tissue secreted significantly less progesterone than ewe lambs tissue with exceptional case of progesterone levels after 6 h at 0 and 10 ng/LH. With the increase of the time of incubation the tissue becomes exhausted, therefore, progesterone concentration was decreased in both ages. The decrease in progesterone secreted from ewes luteal tissue could be described by an increase in the total number of large luteal cells in ewe granulosa cells compared with that in ewe lambs, which affects progesterone secreting in culture media and also the receptors of LH in granulosa layer are less in ewe lambs CL than in adult ewes which is responsible for luteinisation (Carson *et al.*, 1979a). However, there is (Bramley *et al.*, 2005) more conversion of small luteal cells, to large luteal cells which then can secrete higher concentrations of progesterone (Farin *et al.*, 1988). In this experiment the time was limited to occur. Additionally, progesterone concentration showed a rapid increase in the plasma of ewes treated exogenously with oLH on day 3 or day 11 of the luteal phase during the breeding season (Bramley *et al.*, 2005).

The data for luteal tissue cultured in TCM-199 with hCG showed progesterone concentration was significantly higher in ewes luteal tissue than ewe lambs tissue as a response to the hCG (Figure.4.9). The function of hCG is like LH activity and may be caused by luteotrophic stimulation to ewes luteal tissue more than in ewe lambs (Khan *et al.*, 2007). The decline of progesterone concentration after 2 h of incubation give an indication of tissue exhausted after 2 h, which in previousl confirmed by Bramely *et al* (Bramley *et al.*, 2004) when treated ewes CL with hCG. Whilst these findings are in contrast with the results found, that progesterone concentrations were increased with the increase of time incubation (Bramley *et al.*, 2005). These differences between the two ages might reflect the luteal sensitivity to hCG *in vitro* as well as inadequate ewe lambs luteal function. The differences between ewes and ewe lambs, might reflect the luteal receptors and their sensitivity to hCG *in vitro* and inadequate ewe lamb luteal function, which agrees with Bramley et al (Bramley *et al.*, 2004).

Overall protein analysis showed that, ewe gels contain more protein spots than ewe lamb gels, in both treated and control follicular shells. In ewe and ewe lamb gels the majority of treated follicular gels contained more protein spots than the control gels. A total of 109 spots were detected in ewes small treated follicular gel which were absent in the control gel (unique spots) and 35 spots in control gel were absent the treatment gel. Compared with ewe lamb gels, where 38 spots detected in treated gel compared to 37 spots detected in control gel. The ewes medium control gel contained more spots than in the treatment gel, with 128 unique spots compare with 54 unique spots in the treatment gel and ewe lamb gels 54 unique protein spots were detected in treatment gel compare with 53 unique spots in the control gel. The same differences were found in the large gels of ewes and ewe lambs. Large follicles assumed to produce large amount of oestradiol and then progesterone, because it contains a large amount of granulosa

and theca cells than small follicles. The ewes large control gel contained 110 protein spots more than the ewe lambs control gel and 165 protein spots in ewes large treated gel compared with the ewe lambs treated gel. These results show evidence to a high response of ewes follicular tissue to FSH and LH compared with ewe lamb follicular tissue when treated in the same conditions. Furthermore, unique protein spots were estimated as 128 spots in ewe treated large follicular shells, where in ewe lamb gel 92 of protein spots were recorded. In addition the most abundance and large protein spots in ewe large gel were estimated at 30 to 100 MW and in ewe lambs gel estimated at 14.4 to 66 MW. Moreover, the size of protein spots tended to be small with the decrease of MW. In this experiment appeared that the range of MW (30 and 100 kDa) has physiological effects and therefore in ewe follicles produced more steroids than ewe lamb follicles. This discrepancy in spot number according to follicle size is in contrast with Driancourt *et al.*(1996) reported that number of spots not affected by follicles size and oestradiol and testosterone production of each Fec^B Fec^B follicle was correlated with the intensities of the spots of its pattern. However, this study used high fecundity ewes.

The abundance of protein spots in treated gels compare with untreated gels in ewes and ewe lamb gels, also in ewe gels compare with ewe lamb gels has a positive correlation with steroidogenesis, as a result to that the amount of steroidogenic enzymes present in the follicular shells of ewes and consequently theca and granulosa cells produce more steroids. That might confirm the hormone results found earlier in this chapter, that ewe follicles produced more oestradiol and progesterone than ewe lamb follicles in agreement with Driancourt (2001b) who reported that, in calf oestradiol output increase with the age. Furthermore, in ewe different gels up-regulated 2-fold and unique spots estimated at 30 to 100 kDa where in ewe lamb gels were between 14.4 to 97 kDa. Driancourt *et al.*(1996), when cultured whole large ewe follicles

before used for 2 D PAGE, reported that the molecular masses of the majority of proteins spots were estimated at 45 and 30 kDa. This range of molecular weight may has a high expression of the key steroidgenic enzyme, cytochrome P450 17-hydroxylase (CYP17) at this period of oestrous cycle (d 9-12) (MacLaughlin *et al.*, 2007). In the sheep, full expression of steroidogenic enzymes P450_{arom} and P450_{17 α} is acquired when the follicular diameter reaches 3.5mm (Huet *et al.*, 1997) and the decrease in the P450_{arom} and P450_{17 α} ratio in early atretic follicles may be responsible for the early decrease in the tranfollicular oestradiol: testosterone ratio during follicular degeneration (Moor *et al.*, 1978).

4.5. Conclusion

Treatment with FSH and LH increased follicular oestradiol and progesterone production in ewes more than ewe lambs, indicate ewe follicles were more response to gonadotrophins than ewe lamb follicles *in vitro*. There was a decreased in progesterone production from ewe follicles compared with ewe lamb follicles after culture for 24 h with or without FSH and LH, which may indicate that ewe follicles were becomes atretic. Luteal function *in vitro* was greater in ewes than in ewe lambs when stimulated. With gonadotrophins protein analysis showed ewe follicles responded more to FSH and LH, compared with ewe lambs as there were more spots in ewe compared with ewe lamb gels.

Chapter Five

***In vitro* steroidogenic function and proteomics of medium and large size follicles from ewes and ewe lambs treated with progesterone and gonadotrophins**

5.1 Introduction

Several investigations have been conducted using different methods on ewe lambs to improve their reproductive efficiency. These studies concluded that a majority of animals have regular oestrous cycles and normal fertilisation rates, however, ovulation rate and embryo survival were lower in ewe lambs than in ewes (Beck and Davies, 1994a, Davies and Beck, 1993, Khan *et al.*, 2007, Khan *et al.*, 2006). Blood hormones profiles, indicated that LH and progesterone concentrations were lower during the oestrous cycle and early pregnancy in ewe lambs compared to ewes (Davies and Beck, 1993, Khan *et al.*, 2007). Khan *et al.*, (2007) demonstrated that ewe lambs follicles and corpora lutea are less responsive, in terms of steroid hormone secretion, to gonadotrophin challenge than those of ewes during the oestrous cycle and early pregnancy. The above results suggest that follicular maturation and subsequent luteal development are subnormal in ewe lambs. Therefore this experiment was conducted to determine whether progesterone and gonadotrophin supplementation can be used to stimulate normal follicular growth and function in ewe lambs when compared to ewes given an identical treatment. Furthermore, pre and post-maturation follicles were collected post slaughter from ewe lambs and ewes and cultured *in vitro* to minimise any environmental effects on steroid hormone production.

In this experiment, ewes and ewe lambs were fitted with CIDRs to produce normal luteal phase progesterone concentrations where treated with ovagen (ovine FSH) and chorulon (hCG) to induce pre-ovulatory follicular growth and final maturation similar to that induced by FSH and the LH surge *in vivo*. The CIDRs function was to give the same amounts of the progesterone to the both groups age at the same time of the oestrous cycle and to stop the endogenous

progesterone. In addition to measuring *in vitro* steroidogenesis, follicles were subjected to proteomic analysis to determine any differences in protein profile.

5.2 Materials and Methods.

5.2.1 Animals.

Ten ewes and ten ewe lambs were randomly selected and divided into two groups each consisting of five ewes and five ewe lambs each group. Body weight and condition score were individually recorded before starting. Average of condition score was 3 and 2.5 for ewes and ewe lambs respectively (appendix.2. D). Animals were housed in separate pens.

5.2.2 Treatment 1: Ewes and ewe lambs treated with FSH (Ovagen) to induce folliculogenesis.

Five ewes and 5 ewe lambs were marked by green colour on their shoulder. On Day 1 of the experiment, intervaginal CIDR (EAZI-BREED CIDR, International, Pharmacia & Upjohn Limited company) were inserted into the all animals. On Day 10, 11 and 12, animals were i.m injected twice daily with ovagen (follicle stimulating hormone ovine pituitary extract (ICP Biotechnology Ltd. New Zealand). Ovagen doses were calculated on body weight. (1.2 ml for ewes and 0.8 ml for ewe lambs).

On day ten of the experiment, the CIDRs were replaced with new CIDRs, to maintain blood progesterone concentrations. On the morning of Day 13, CIDRs were removed and animals were injected in the jugular vein with 20 ml of Euthala (200mg Pentobarbital sodium ph Eur IN each ml: Vericore Ltd, UK). Immediately after the animals were euthanized, ovaries from each animal were collected using sterile forceps and scalpel blade. These were submerged in warm PBS (37°C) with antibiotics in separate in isolated containers for each age groups and transferred to the laboratory within 1 hour of euthanasia.

5.2.3 Treatment 2: Ewes and ewe lambs treated with ovagen and hCG to induce follicular growth and maturation

The second group of animals (5 ewes and 5 ewe lambs) were kept in a separate pen. CIDRs were inserted on Day 1 and changed on day 10 as described in section 5.2.2. From Day 10 until Day 12 animals were treated as described in section 5.2.2. On Day 12, after the last ovagen injection the CIDRs were removed and discarded. On Day 13, 3 iu. per kg of Chorulon (hCG) (Chornic Gonadotrophin, Intervet UK Ltd) was injected to each animal. The dose was calculated by body weight (210 iu to ewe and 120 iu for ewe lambs). Eight hours after hCG injection, all the animals were euthanized as described on section 5.2.2. The ovaries were collected and treated as section 5.2.2.

5.2.4. Blood samples

Before sampling, the neck area around the jugular vein was trimmed (about 20 cm²). Five ml of blood was taken from each animal. Samples were taken over four days, starting from first day of Ovagen injection (Day 10) until euthanasia. (Day 13) The last blood was taken prior to hCG injection. Blood samples were collected each day at 9:30 am and brought back to the laboratory within 30 mins of collection. The blood samples were taken from the jugular vein by venipuncture in heparinised tubes (Sherwood Medical, Gosport, UK) and centrifuged for 10 mins at 3000 rpm at room temperature. Plasma was then separated by 1ml pipette and collected in a small tube container and immediately frozen at -20°C for hormone assays. Progesterone concentrations for both treatment groups were assayed at the same time by RIA, as described in section 2.5.1.

5.2.5 Follicle culture

Follicles from both groups of animals were dissected from the ovaries from sheep of both treatments groups and then classified as described in section 2.4.1. Medium and large follicles were approximately cut into two halves by sterile forceps and scalpel blade before being washed three times in warm TCM-199. One half was stored at - 80°C, and the other was cultured in 2ml of TCM-199 with antibiotics. Small follicles were grouped as 5 follicles per group, washed three times in TCM-199 before being cultured. Follicular fluid, oocytes and cumulus cells were kept at - 80°C for protein electrophoresis. Follicles for culture were incubated at 37°C in 95 % air and 5% CO₂. Total number of cultured follicles of first treatment (ovagen) is shown in Table 5.1 and for second treatment group in Table.5.2.

Culture media were changed every 2 h for 8 h and stored at - 20°C until oestradiol and progesterone were assayed. From each age group, 8 follicles media from both (medium and large) follicle sizes were randomly chosen for oestradiol assay and 10 for progesterone assay.

Follicular shells were weighed in order to calculate hormone secreted per milligram of tissue. The oocytes were collected and cultured as described in section 6.2.1. There was no functional CL identified. Oestradiol and progesterone levels were determined in follicle culture media and in the serum samples using RIA method as described in section 2.5.1 and 2.5.2.

5.2.6 Proteomics

Three identical replicate gels were run for each age group within each treatment group. Halves of the medium follicle shells (n = 12) from each age and treatment group were used to prepare PAGE samples. Before casting gels, a preliminary experiment was done using follicular shells of both ages they were run in 2DE using 17 cm IPG-Strips pI 3 to 10. The result from that

showed that the majority of protein spots were ranged between *pI* 5 to 8 in both ages. Therefore, IPG-Strips with *pI* 5-8 were considered to be used for optimum results. Sample preparation was done as described in section 3.2.3.1.1 and protein purification was done using Tri-chloro acetic acid in acetone method as described in section 2.6.1.1. The whole process of one and two dimension electrophoresis was followed as described in section 3.2.3.1.2. Protein spots detection was done using progenesis software as described in section 2.7.4 following storage at 4°C in sandwich plastic.

5.2.7 Data analysis

Oestradiol and progesterone in follicles media were expressed as ng/mg and analyzed by split-plot design using GenStat 8. The data from blood samples were analyzed as ng/ml by general analysis of variance, in order to be in normal distribution all data were used after log transformation.

5.3. Results

5.3.1 Treatment 1: Ewes and ewe lambs treated with Ovagen

5.3.1.1 Oestradiol concentrations in culture media of follicles.

The number of ovaries and follicles from each age of animals are shown in (Table.5.1). There was no significant ($P>0.05$) difference in mean oestradiol concentrations in media between ewes and ewe lambs follicles at each sample time was found (Figure.5.1 and Appendix.5.1). Moreover, there was no difference ($P>0.05$) in oestradiol concentration between medium and large follicles in either ewes or ewe lambs, mean log medium was log 0.35 pg/mg and large follicles oestradiol was 0.26 pg/mg (SED = 0.07).

Table.5.1 The number of animals, ovaries and follicles size of treated ewes and ewe lambs with ovagen. Small follicles cultured as group of five follicles each.

	No: of animals	No: of ovaries	Large follicles	Medium follicles	Small follicles
Ewes	5	10	24	12	10
Ewe lambs	5	10	12	8	10

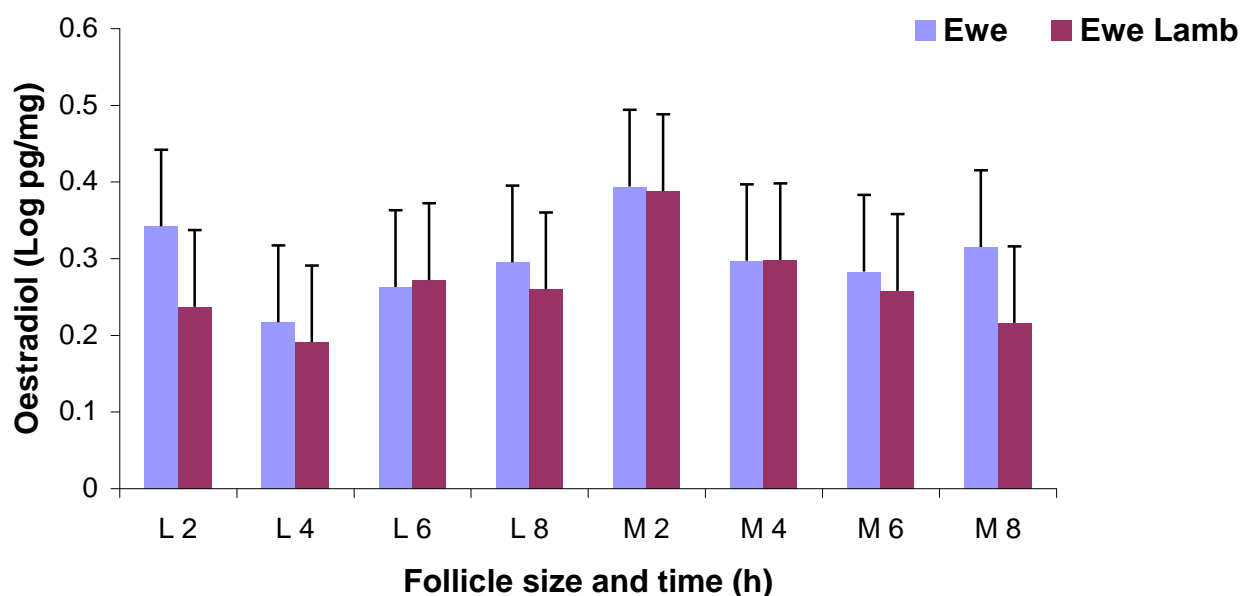


Figure.5.1 Effect of length of incubation on mean media oestradiol concentration in medium and large ewe and ewe lamb follicles treated with ovagen (SED = 0.1). L: large, M: medium, 2, 4, 6 and 8 h =hours (n = 32 follicles/time per age).

5.3.1.2 Treatment 1 Progesterone concentrations in culture media of follicles.

The results of this experiment showed no difference between ewe and ewe lamb follicles in progesterone concentrations ($P > 0.05$). However, progesterone concentration decreased with increase incubation time in ewe and ewe lamb medium follicles (Figure.5.2). Overall mean log progesterone in ewes medium and large follicle media was 0.29 ng/mg and in ewe lambs 0.26 ng/mg (SED = 0.016). Furthermore, no differences ($P > 0.05$) were found in progesterone concentrations between ewes and ewe lambs over time (Figure.5.2).

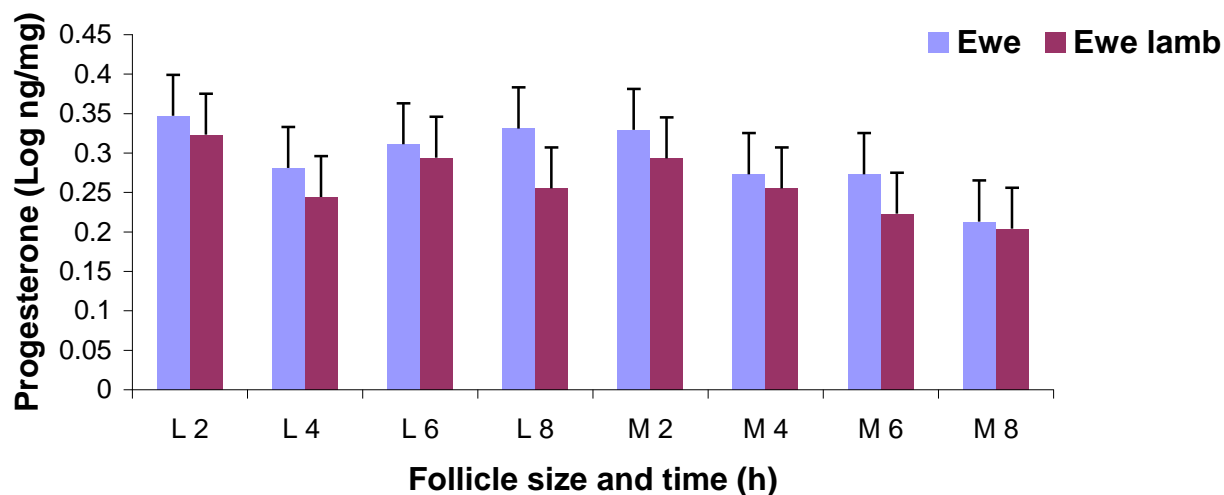


Figure.5.2 Effect of time of incubation on progesterone concentration from ewe and ewe lamb half follicles treated with ovagen (SED = 0.052). L: large follicle size, M: medium follicle size. 2, 4, 6 and 8 h = hours (n = 32 follicles/time per age).

5.3.1.3 Blood progesterone levels for ovagen group

There was no difference ($P > 0.05$) between ewes and ewe lambs in overall mean plasma progesterone concentrations. However, a significant difference ($P < 0.05$) was observed in progesterone concentration between times of sample collection as progesterone concentration gradually increased with time (Figure.5.3). For example, progesterone concentrations were higher on the Day 11 than Day 10, and higher Day 12 than on Day 11 in both groups of animals (Figure.5.3 and Appendix.5.2). On day 13, progesterone concentrations were significantly ($P < 0.05$) lower than on day 12. Furthermore, progesterone concentration on Day 10 was significantly ($P < 0.05$) higher in ewes than in ewe lambs. No interaction between age and time was observed.

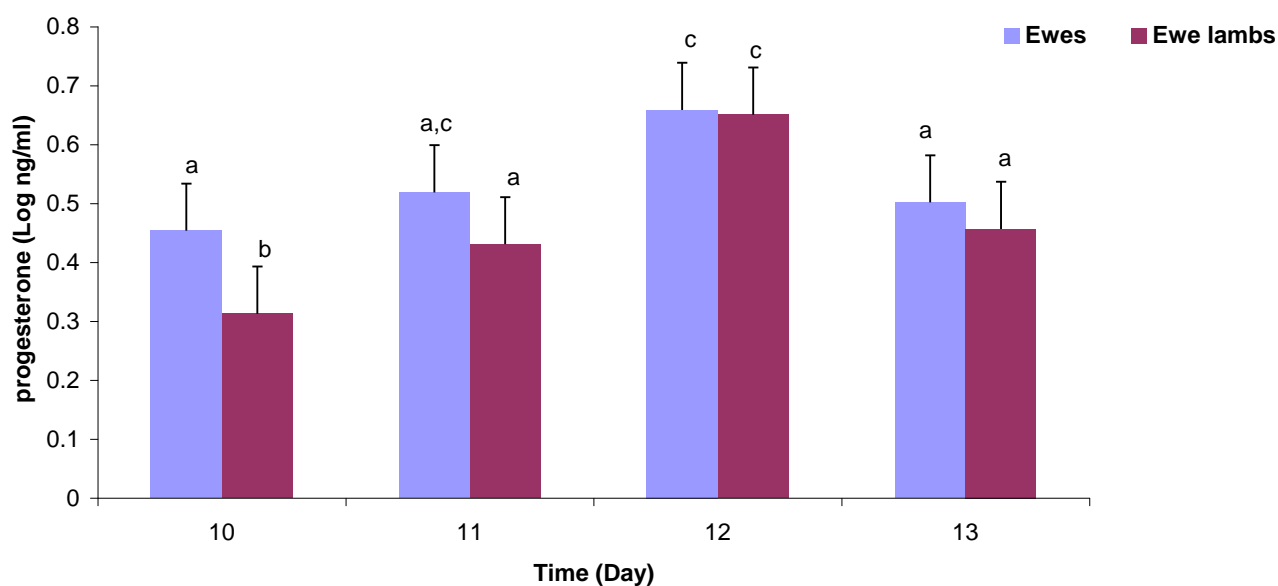


Figure.5.3 Effect of time and treatment on progesterone produced from ewe and ewe lamb treated with ovagen blood samples (SED = 0.08). Bars with different letters are significantly different (n = 5 samples per age each day).

5.3.1.4. Treatment 1: Proteomics of follicular shell from ewes and ewe lambs treated with ovagen

The comparison between ewes and ewe lambs average gels was done using progenesis software. The percentage overlap between ewes' three replicate gels was 70% and between ewe lamb three replicate gels was 47%. The results from this experiment showed that ewes average gel had less up regulated spots than the average from ewe lambs (Table.5.2) and more down regulated spots in the ewes gel than the ewe lambs gel. Most abundant spots in both gels were estimated at 30.1 to 97 kDa marker and ranging between *pI* 5 to 8 (Figure.5.4 A and B). Up regulated and down regulated 2-fold spots in both gels were estimated at 30 to 97 kDa marker and ranging between 5 to 8 *pI*, for example for ewes up regulated protein spot 95 show an increase 2.68-fold and for down a regulated protein spot, 105 was shows a decrease 2.65-

fold (Figure.5.5A and B). Furthermore, unique spots were estimated at the same MW marker of up and down regulated spots in both gels (Figure.5.6).

Table.5.2 Medium follicular shells of ewe and ewe lamb treated with ovagen

Spots	Lambs ovagen	Ewes ovagen
Total number of spots	253	302
Matched %	66	100
Up regulated 2 fold	77	40
Down regulated 2 fold	40	77
Between \pm 2 fold	84	119
Unique spots	52	66

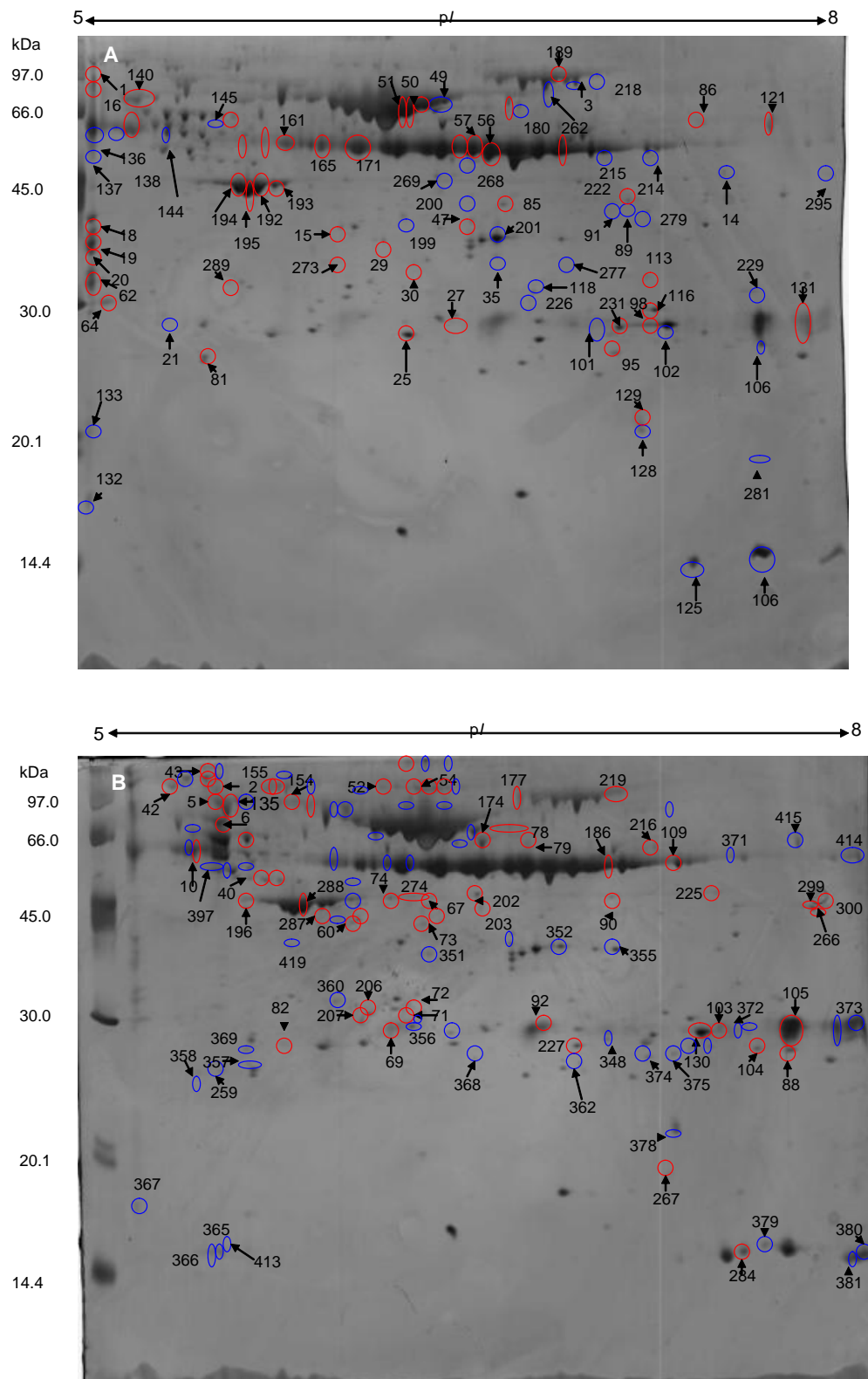


Figure.5.4 Pattern of protein secreted by ewe medium follicular shells treated with ovagen **(A)** and pattern of protein secreted by ewe lamb medium follicular shells treated with ovagen **B**. Up-regulated spots (red) and unique spots (blue). Spots without circle are less than 2-fold.

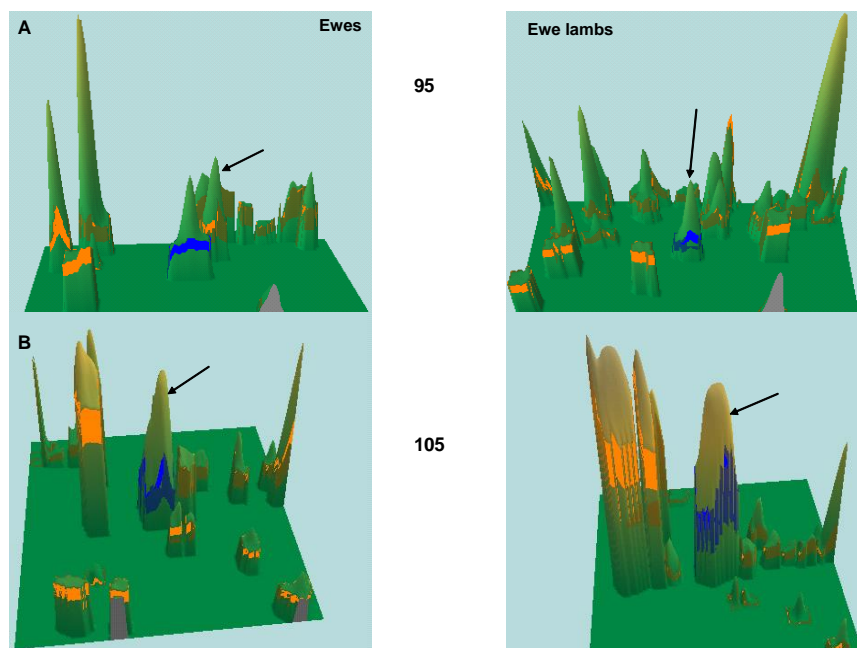


Figure.5.5 Three dimension image of spot 95 up-regulated 2.68-fold ewe average gel (A) and spot 105 down regulated 2.63-folds (B) compared with the same spots in ewe lamb average gel from medium follicular shells from ewes and ewe lambs treated with ovagen.

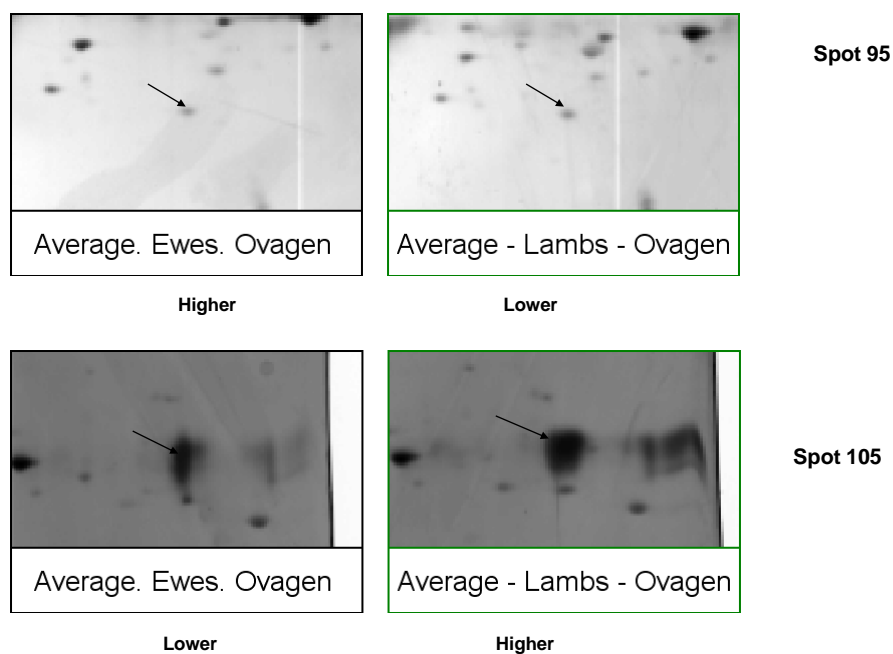


Figure.5.5 B Montage images for up (95) and down regulated (105) spots from average gels of medium follicular shells from ewes and ewe lambs treated with ovagen.

5.3.2 Treatment.2: Ewes and ewe lambs treated with ovagen plus hCG

5.3.2.1 Oestradiol concentrations in media

The number of animals, ovaries and follicles collected from ewes and ewe lambs are shown in Table.5.3. In over all oestradiol concentrations in follicles media of ewes treated with ovagen plus hCG were significantly ($P<0.05$) higher compared ewe lamb follicles media. Mean log of ewes oestradiol concentration was 0.73 pg/mg and ewe lambs log mean was 0.5 pg/mg (SED = 0.068). Furthermore, oestradiol concentrations tended to decrease gradually with increased time of incubation, but that decrease was not significant ($P>0.05$). Furthermore, there was no interaction between age, time and follicle size (Figure.5.6).

Table.5.3 The number of animals, ovaries and follicles from treated ewe and ewe lamb with ovagen plus hCG. Small follicles cultured as group of five each.

	No: of animals	No: of ovaries	Large follicles	Medium follicles	Small follicles
Ewes	5	10	24	14	5
Ewe lambs	5	10	17	12	10

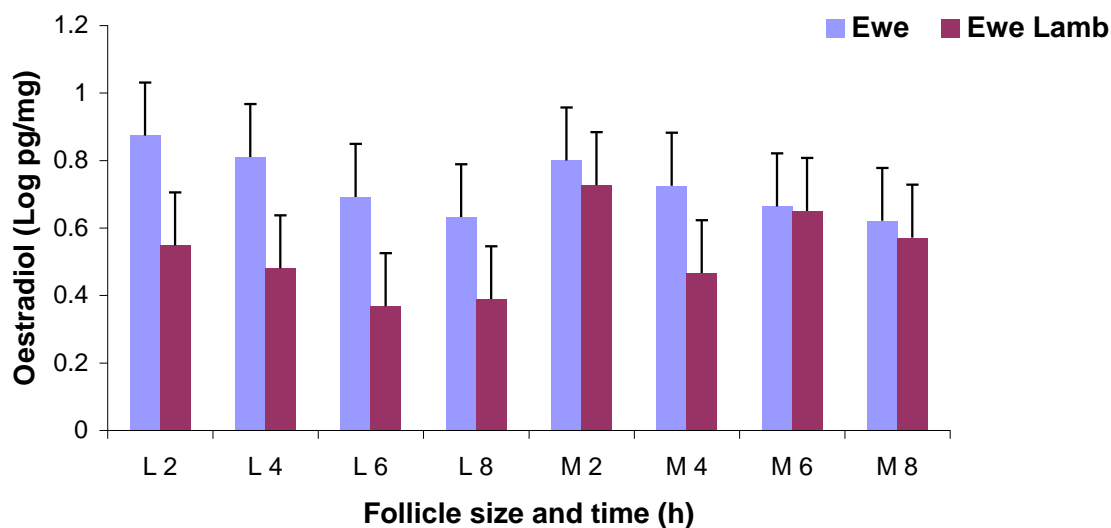


Figure.5.6 Effect of length of incubation on mean oestradiol concentration in ewe and ewe lamb follicles treated with ovagen + hCG (SED = 0.15). L: large follicle size, M: medium follicle size. 2, 4, 6 and 8 h = hours (n = 32 follicles/time per age).

5.3.2.2 Treatment 2 Progesterone concentrations in media

The results from this experiment found was no statistical differences ($P > 0.05$) in progesterone concentration between ewe and ewe lamb in follicles media, mean log progesterone concentration of ewe was 0.296 ng/mg and ewe lamb was 0.257 ng/mg (SED = 0.026). Progesterone concentration in medium follicles was decreased with increasing time of incubation in both ewes and ewe lambs. However, progesterone from large follicles decreased after 2 h and then similar concentrations of progesterone was secreted for the remaining times of incubation (Figure.5.7). However, progesterone concentrations in large follicles media was significantly ($P < 0.05$) higher than in medium follicles media, mean log progesterone from large follicles was 0.299 ng/mg and in medium follicles was 0.254 ng/mg (SED = .02).

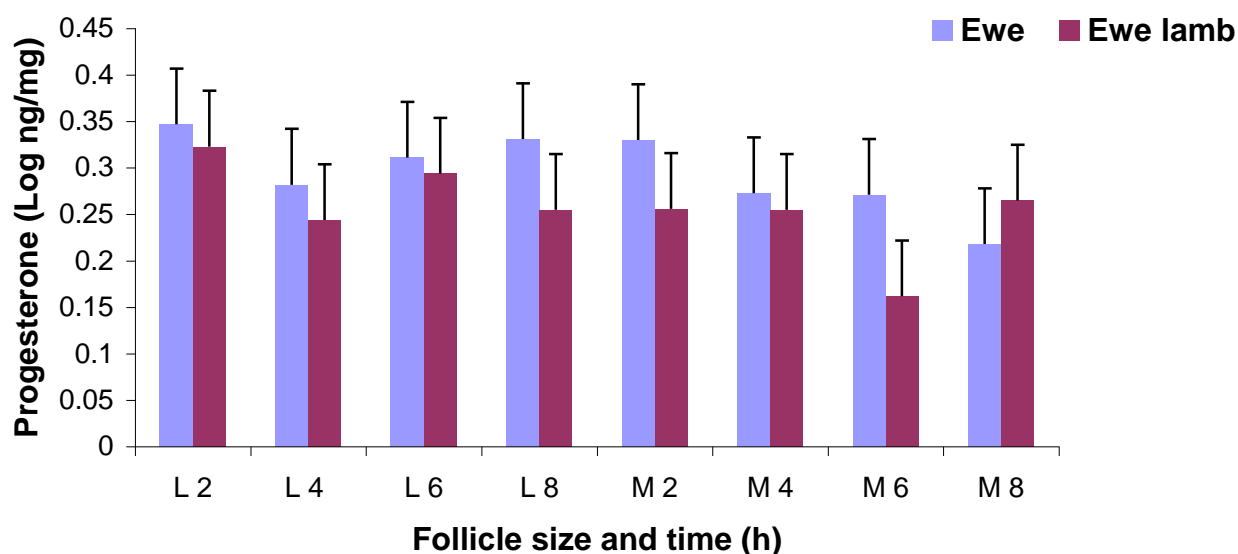


Figure.5.7 Effect of length of incubation and age on ewe and ewe lamb follicles treated with ovagen plus hCG (SED = (0.065). L: large follicle size, M: medium follicle size. 2, 4, 6 and 8 h = hours.

5.3.2.3 Blood progesterone levels for ovagen plus hCG group

Overall progesterone concentration was significantly ($P < 0.05$) greater in ewe samples compared with ewe lamb, overall mean log progesterone concentration was 0.466 ng/ml and in ewe lambs was 0.394 ng/ml (SED = 0.024). Moreover, in both ewe and ewe lamb samples progesterone levels increased from Day 10 to Day 12 and decreased on Day 13 (Figure.5.8), but this increase was not significant. No statistical differences found on the interaction between age and time (Appendix.5.3)

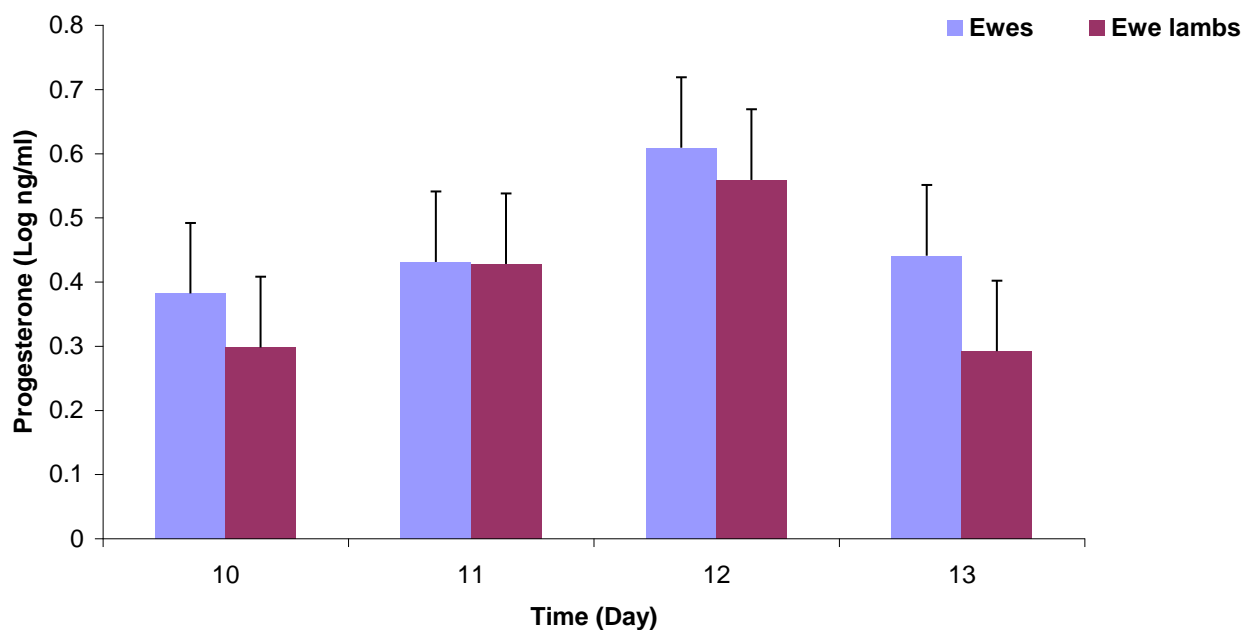


Figure.5.8 Effect of time and treatment time on progesterone concentrations in blood sample of ewes and ewe lambs treated with ovagen plus hCG (SED = 0.09) (n = 5 samples per age each day).

5.3.2.4 Treatment.2 Proteomics of follicular shells from ewes and ewe lambs treated with ovagen plus hCG

Data for medium follicular shells of ewes and ewe lambs treated with ovagen plus hCG showed more protein spots in ewe lamb average gel than ewe gel (Table.5.4). Moreover, a good matching percent (60%) was observed between the two gels when the ewe average gel was used as control. The identical percentage between ewe three replicate gels was 60% and between ewe lambs three replicate gels was 59 %. The results from this experiment showed little difference between the gels in terms of the number of spots that were up and down regulated, though the gels for the ewes contained more up regulated spots (58) than the gels from ewe lambs (54) (Table.5.4). Furthermore, the most abundant spots in both gels were estimated at 30 to 100 kDa marker and ranging between pI 5 to 8 (Figure.5.9 A and B). Up

regulated and down regulated 2-fold spots in both gels were estimated at 30 to 45 kDa marker and ranging between 5 to 8 pI, for example for ewes up regulated protein spot 199 shown an increase 2.74-fold and for down regulated protein spot 74 was shown a decrease 2.41-fold (Figure.5.10 A and B). Furthermore, unique spots were estimated at the same MW marker of up and down regulated spots in both gels (Figure.5.9). The full results tables for all proteomics experiments for this chapter shown in the CD (appendix.5).

Table.5.4 Medium follicular shells of ewes and ewe lambs treated with ovagen plus hCG.

Spots	Lambs hCG	Ewe hCG
Total number of spots	277	291
Matched %	60	100
Up regulated 2 fold	54	58
Down regulated 2 fold	58	54
Between \pm 2 fold	110	86
Unique spots	55	93

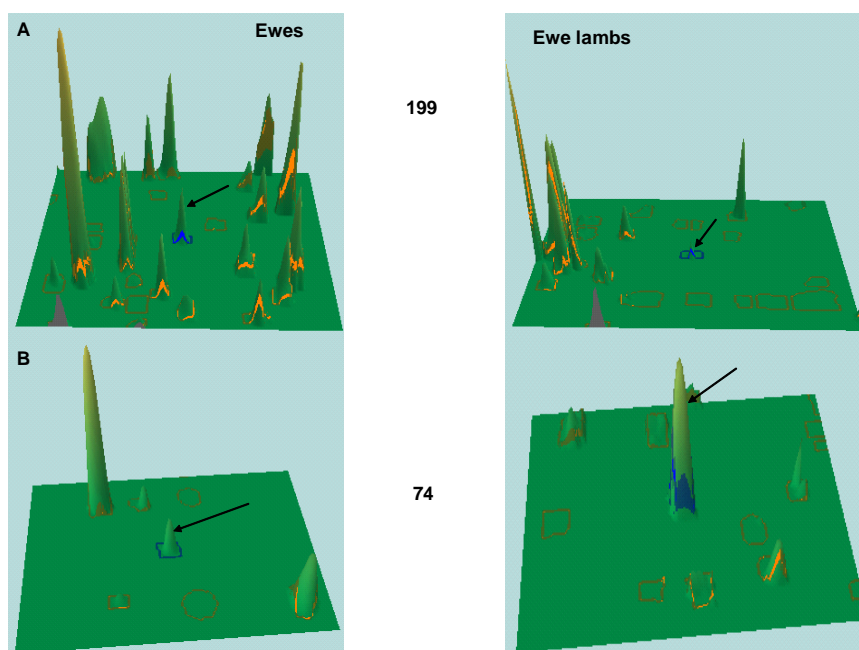


Figure.5.10 A Three dimension image of spot 199 up-regulated 2.74-fold ewe average gel (**A**) and spot 74 down regulated 2.41-folds (**B**) compared with the same spots in ewe lamb average gel from medium follicular shells from ewes and ewe lambs treated with ovagen plus hCG.

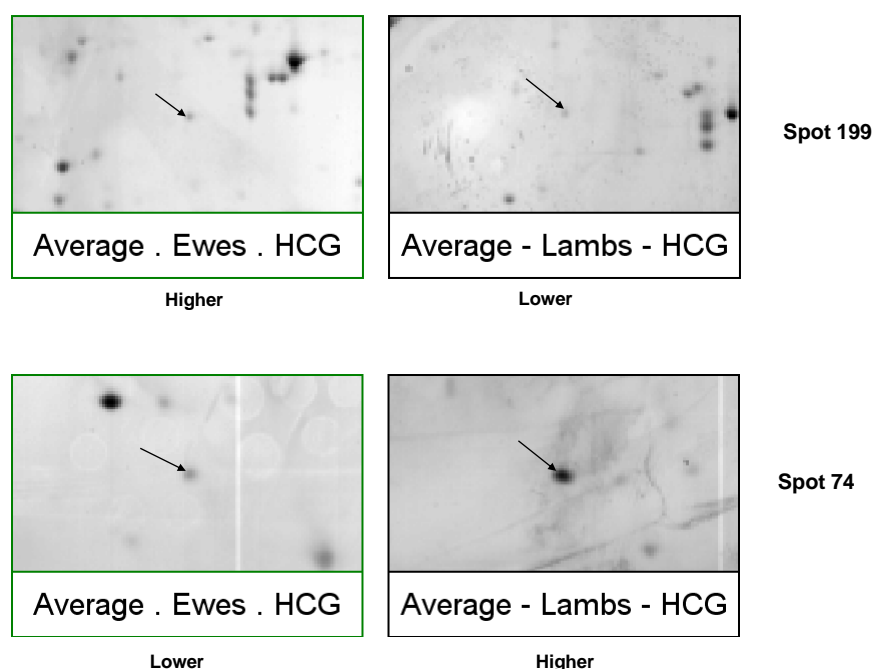


Figure.5.11 B Montage images for up (199) and down regulated (74) spots from average gels of medium follicular shells from ewes and ewe lambs treated with ovagen plus hCG.

5.4 Discussion

Ewe lamb ovaries are less responsive than adult ewe ovaries in terms of follicle development, which agrees with earlier studies showing low ovulation rate in ewe lambs (Davies and Beck, 1993, Dyrmundsson, 1973a, Dyrmundsson, 1983). The results of oestradiol in media from follicles from treated animals with ovagen revealed that oestradiol concentrations were the same as the follicular media of treated ewes and ewe lambs with ovagen (Figure.5.1). this results showed that oestradiol concentrations was increased with the increasing follicle size in sheep (Mann *et al.*, 1992, Moor *et al.*, 1973, Webb *et al.*, 1989) and mares (Beck *et al.*, 2003). This is also similar to that found between cow and calf reported by Driancourt *et al.* (2001b) who compared oestradiol production between adult and young cattle *in vitro*. The similar concentrations of oestradiol in both age groups in this experiment could reflect the response of these follicles to oFSH. Medium and large follicles size would have a similar number of cells in the theca and granulosa layers, therefore, the same quantity of cholesterol should be converted to oestradiol in follicular shells from both ages.

The results of progesterone in follicles from groups treated with ovagen show that there was no difference in media progesterone concentrations in follicles from ewes and ewe lambs treated for four days with ovagen, or between different times of incubation within the age were found. This suggests the enzymatic activity in ewes and ewe lambs theca and granulosa layers were similar for the same size follicles. This contrasts with Tambe and Nandedkar (1993) who reported that progesterone concentrations were higher in follicles from ewes than that from ewe lambs. Furthermore, hormone production after 2 h of incubation reflects *in vivo* steroid production, and follicular fluid steroid concentration (Webb and Gauld, 1987).

Treatment with ovagen plus hCG showed significantly increased oestradiol secretion from ewes follicles more than ewe lambs follicles. This experiment confirmed the findings in Chapter 4 when follicles were cultured with FSH/LH. The increase in oestradiol concentration was due to hCG action, which is similar to that of LH. It is possible that the lower response in lambs to hCG was because, thecal cells are incapable to producing sufficient amounts of androgens as substrate for oestradiol synthesis by lambs granulosa cells. Consequently, intrafollicular concentrations of oestradiol are lower. The oestradiol concentration decreased with increased incubation time, may mean follicular tissue become atretic with time. This agree with Moor *et al.* (1973) who showed that short exposure to gonadotrophic hormones stimulates oestrogen secretion by ovarian follicles. Also Driancourt *et al.* (2001b) reported that oestradiol concentration increased as the calves grew older. Furthermore, exogenous treatment with GnRH or hCG increased oestradiol concentrations. This agrees with *in vivo* work showing ewe ovaries respond more to GnRH/hCG than ewe lambs, which can explained by more follicles present in the ewe ovaries, thus producing more oestradiol (Khan *et al.*, 2007, Khan *et al.*, 2006). The lower oestradiol in ewe lamb follicles media is due to fewer responsive follicles compared with ewe.

The results of progesterone from treated animals with ovagen plus hCG showed that ewe follicles produced more progesterone in media than ewe lamb follicles. Furthermore, progesterone concentration decreased with increased incubation time. These results indicate that ewe follicles responded to ovagen more than those in ewe lambs, and also the effect hCG, which has the same action as LH in ewe lambs was less than in ewe follicles. Activated luteinized granulosa cells may be present in greater number in ewes than in ewe lambs and subsequently produce more progesterone. This could due to more active steroidogenic

enzymes, which convert cholesterol to progesterone (CYP11A: cytochrome P450 side chain cleavage; CYP17: cytochrome; 3 β -HSD: 3 β -hydroxysteroid), in ewes theca cells compared with ewe lambs, consequently less cholesterol is converted to progesterone in ewe lambs theca cells. Moreover, it could be due to more progesterone in ewe lambs being converted to oestradiol, so less progesterone is present as steroidogenic enzymes necessary for progesterone synthesis. In addition to which the majority of binding sites for FSH and mRNA for FSH receptor were localized in membrane of ewe granulosa cells more than in lamb granulosa cells.

In the ewes and ewe lambs given CIDRs to supply progesterone, the ewes should have the same plasma concentrations as ewe lambs. However, concentrations were greater in ewes which may mean that the absorption area in ewes vagina are greater than in ewe lambs, lambs clearance rate is higher or ewes have more endogenous progesterone.

The results from the ovagen treated groups showed that plasma progesterone concentrations were increased with increasing the days of treatment in both ewes and ewe lambs. Whereas, no difference found between two ages of groups (Figure.5.5). These results are in contrast to those found between ewes and ewe lambs. After oestrus, synchronization with progesterone sponges and eCG on day 12 of pregnancy and 4 μ g GnRH or 200 IU hCG on day 12 post matings , Khan *et al.* (2007) reported that progesterone concentration was higher in ewes compared with ewe lambs. Ewe lambs progesterone levels increased as a result of treatment, but not as much as that in ewes. In this experiment, the same response from the two ages to the sponge and oFSH effect may be due to the same number of FSH receptors in both ewes and ewe lambs ovaries.

The results from animals treated with ovagen plus hCG showed an increase in progesterone levels of ewe samples compared with lambs and an increase in progesterone with treatment time with ovagen in both ewes and ewe lambs (Figure.5.8). However, a decrease was observed in both age groups on the day injected with hCG. Moreover, steroidogenic enzymes like CYP11A, CYP17, 3 β -HSD in ewe theca cells layer were more active compared to ewe lambs. Also in ewe lambs, low levels of progesterone may reflect increased metabolic clearance. It is unlikely that progesterone from follicles could affect plasma progesterone concentration.

The experiment for protein analysis of ovagen groups was designed in order to investigate protein synthesis in ewes and ewe lambs follicular shells. In total 302 and 253 protein spots were found respectively in ewes and ewe lambs master gels. Only 47 % of lamb protein spots were matched with ewe average gel, with 66 and 52 unique protein spots respectively in ewes and ewe lambs. The major abundance of upregulated 2-fold and unique protein spots were estimated to be between 30 to 66 kDa were similar in both ewes and ewe lambs gels. Whilst the main difference was in the number of upregulated (40 versus 77) and unique spots (66 versus 52) in ewes and ewe lambs respectively. The lowest percentage (47%) of matching spots of ewe lambs average gel with ewe average gel has affected the protein differences between the average gels for age groups.

From the results of ovagen plus hCG treated groups showed that the response of ewes and lambs to ovagen plus hCG were lower compared with ovagen only groups. As a lower number of protein spots were counted in both ewe and ewe lamb average gels in comparison with the previous experiment (291 and 277) total protein spots and 93 and 55 unique spots in ewes and ewe lambs gels respectively. Abundant protein spots were estimated between 30 to 45 kDa

marker. More protein spots in ewe average gel could indicate to new protein in ewes in response to hCG. From the results of both treatments (with ovagen and ovagen plus hCG) protein spots between 30 to 66 MW related to steroidogenic synthesis.

5.5 Conclusion

This experiment found that, treatment with ovagen increased oestradiol concentration in both ewes and ewe lambs similar levels and also the same hormone level was obtained from medium and large follicles, but treatment with ovagen plus hCG increased ewe oestradiol compared with ewe lamb follicles. In addition, the same results were observed in progesterone concentration in both treated groups. Plasma results showed increased progesterone levels with increased treatment time in both treated groups. In the group treated with ovagen plus hCG, progesterone concentration decreased after hCG was injected as a result of stimulate follicles producing more oestradiol than progesterone, via the binding of hCG with LH receptors. Ovagen was also shown to effect protein synthesis.

Chapter Six

A comparison of oocyte maturation *in vitro* in ewe lambs and ewes

6.1 Introduction

The use of juvenile animals as oocyte donors in economically important species would reduce generation interval (Lohuis, 1995, Walmsley, 1996). In sheep the developmental ability of juvenile female oocytes is lower than adults, although the explanations for this vary (Duby *et al.*, 1996, Ptak *et al.*, 1999). Generally, the low competence of prepubertally derived oocytes can not be attributed only to the age because oocytes derived from 1-month old lambs were of similar or higher potential than those from adult oocytes, also a low development potential was associated with improper follicular response following gonadotrophin treatment (Ptak *et al.*, 2003) and the differences between lamb-and adult-derived oocytes, such as the smaller size and patterns of protein synthesis and energy metabolism, contribute to the reduced competency of juvenile-derived embryos (Gandolfi *et al.*, 1998, O'Brien *et al.*, 1996, Ptak *et al.*, 1999).

Calf oocytes exhibited lower fertilization rates after *in vitro* maturation (36%) but their rates of development to morulae and blastocysts did not differ from that of mature oocytes (Armstrong *et al.*, 1992). In calves, the proportion developing to morulae was significantly higher ($P < 0.05$) than that observed for zygotes resulting from *in vitro* maturation and fertilization of oocytes recovered from cow ovaries obtained at an abattoir (Armstrong *et al.*, 1992). Ovaries of pre-pubertal calves exhibit a wavelike pattern of follicular growth, which is similar to those of post pubertal animals (Adams *et al.*, 1994, Evans *et al.*, 1994). Collection of calf oocytes and their *in vitro* maturation, has shown that they can resume meiosis, undergo germinal vesicle breakdown, reach (MII), and can be fertilised but develop into abnormal embryos (Armstrong *et al.*, 1992, Ledda *et al.*, 1999 a and b).

During meiotic maturation, fully grown oocytes undergo ultrastructural and functional modifications that allow them to continue monospermic fertilisation and development (Ducibella et al., 1990). Furthermore, during oocyte growth, physiological events occur in both the nucleus and the cytoplasm allowing the oocyte to achieve final development competence (Ledda *et al.*, 1999b-a). The production of a viable egg depends on the synthesis of mRNA and protein during oocyte growth followed by the complex reorganization of cytoskeleton and organelles in the cytoplasm before the release of the oocyte from the follicle (Kochhar *et al.*, 2002b). In sheep, meiotic and developmental competence is higher in oocytes recovered from large follicles than in those derived from small follicles (Ledda et al., 1997 and Kohhar et Al., 2002). In addition, oocytes cultured in intact follicles (<2 mm) are incapable of resuming meiosis (Moor and Trounson, 1977).

Therefore, the aim of this experiment was to investigate the factors affecting *in vitro* maturation of oocytes, such as age and type of media. Different types of media were used to determine whether the abnormal oocyte development that occurs in ewe lambs *in vivo* can be overcome by *in vitro* culture in follicular fluid from mature ewes. In addition *in vitro* oocyte protein synthesis in ewe lambs and ewes was compared using 1D gel electrophoresis.

6.2 Materials and Methods

Animal management as described in section 2.3, ovaries were collected (ewes n= 226 and ewe lambs n= 279 ovaries) from the animals described in Chapter 3 and Chapter 4. Collected ovaries were brought back to the lab as described in section 2.4 and washed twice in warm PBS with antibiotics and placed in a warm TCM-199 with antibiotics in a sterile flask and placed in a water bath (33°C) in a fume cupboard, to prevent samples of contamination.

Medium and PBS were prepared in plastic containers and incubated for 24 h before use. All subsequent procedures were conducted in a laminar flow workstation (Air flow servicecare. Hampshire. UK), which was sterilised by 70% (v/v) ethanol spray (Sigma Aldrich). A water bath and a binocular microscope were placed in the laminar flow cupboard and turned on 30 mins before starting. This work was conducted in a automatic air conditioned room, the temperature of which was controlled within the range of 25 to 30°C.

6.2.1 Oocytes collection

Ovaries were submerged in warm TCM-199 and placed in a water bath as described in section 6.2. The collection of oocytes was done by slashing ovaries in pre-warm TCM-199 (33°C) supplemented with 100 IU/100ml heparin to prevent clotting. Healthy oocytes which were fully surrounded with at least three layers of cells were collected under the binocular microscope 20 x magnifications using 2µl pipette. Oocytes were transferred to a new petri dish with fresh medium, washed three times with the medium plus heparin. Using a 2µl pipette, all the oocytes were then transferred into a new petri dish with fresh warm medium. All cultures using ewes and ewe lambs oocytes were done in parallel during all the *in vitro* work. During oocyte collection, the medium was changed for fresh medium at 33°C every 30 mins in order to keep the oocytes healthy prior maturation.

6.2.2 Oocyte maturation

Under a binocular microscope and using 2 µl pipette all the oocytes covered with at least 3 complete layers of cumulus cells were transferred into a warm TCM-199 with heparin in a new petri dish. Any naked oocytes were moved to a separate petri dish. Oocytes from each age were separately collected. Ewes follicular fluid, ewe lambs follicular fluid were collected from groups of animals treated with ovagen and ovagen plus hCG which referred in section 5.2.1.1 and 5.2.1.2 and BSA. These additives were diluted in TCM-199 medium to have 50 µl of each medium and stored

as aliquots 0.5ml at -20°C until used. Oocytes were randomly allocated in four groups and matured in multiwell dishes and then cultured in 0.5ml of TCM-199 in groups of 35 oocytes using four well dishes with antibiotics supplemented with 50 µl of one of the following:

- a) FCS serum (v/v)
- b) Ewes follicular fluid.
- c) Ewe lamb follicular fluid.
- d) Bovine serum albumen (BSA)

A total of of 50 µl of both FSH and LH were added to the all groups. The above cultures were performed three times. The total numbers of cultured ewes oocytes were 414 for ewes and 411 for ewe lambs and the number of oocytes was collected per group as showed in (Table.6.1).

Tabl.6.1 Number of ewe and ewe lamb oocytes cultured in four different media. FCS fetal calf serum, E F F ewes follicular fluid, EL F F ewe lambs follicular fluid, BSA: bovine serum albumin

	FCS	E F F	EL F F	BSA
Ewes	105	102	103	105
Ewe lambs	105	105	101	100

Before incubation, all groups were monitored by microscope (Leica Microsystems Wetzlar GmbH, Wetzlar Germany) with 100 x magnifications and random oocyte images were captured (from each group within two minutes) from each treatment in order to determine cumulus and cytoplasm area prior to maturation using Leica IM50 software Version 6. (Leica Mirosystemsystem Digital Imaging. 106, Company© 1992-2003 Imagic Bild verarbeitung AG. Leica Mirosystem Imaging Solutions Ltd. UK). All images were then saved in two files, according to age. Immediately all oocytes dishes were carefully incubated for 24 hours at 38°C in an atmosphere of 5 % CO₂.

6.2.3 Oocyte evaluation

After 24 h of incubation, oocytes were monitored again using the above method to evaluate oocytes in each medium and each age. Five oocytes from each treatment per age per time were randomly assessed for cumulus expansion measuring after maturation. Images were captured as described in section 6.2.1.1 and saved for analysis. Each group of oocytes were then moved to the new four well dishes with 0.5ml of PBS before oocyte staining.

The total surface area of each cytoplasm and cumulus from both ewes and ewe lambs were measured using Axiovision software (Axiovs 40 V 4.6.1, Copyright © 2002-2007card Zeiss Imaging solutions. GmbH). Oocytes were measured before and after maturation for 15 oocytes from each media for each age group described in (Table.6.2). The oocytes were randomly chosen for evaluation and the number of oocytes having reached MII, from each medium per age, was assessed from the presence or absence of a polar body

6.2.4 Oocyte Staining

Fifteen oocytes were randomly chosen from each treatment for staining and nucleus development investigation. After removing the cumulus cells, chosen oocytes were transferred (under binocular microscope) into a clean cavity slide using a 2µl pipette, and 50µl of 1: 100 Bis-Benzimide (Sigma Aldrich) was added. A cover slip was gently pressed down until it touched and secured the oocytes. In a dark area, the slides were incubated for 10 mins, before being ready to observe under the fluorescent microscope. The fluorescent microscope was turned on 15 minutes before use (warmed up before use). In the dark room oocyte maturation was examined under the 20x magnification and filter number 2. The nuclear maturation of the oocyte was examined based on the nuclear morphology and which oocyte had reached the metaphase II stage with presence of a polar body, which meant it is a matured oocyte. The oocytes not used for the above were stored as

groups at - 80°C for electrophoresis. The total number of oocytes stained per group is shown in Table 6.2.

Tabl.6.2 Number of oocytes evaluated and stained after maturation in four different media from ewes and ewe lambs. FCS fetal calf serum, E F F ewes follicular fluid, EL F F ewe lambs follicular fluid, BSA: bovine serum albumin.

	FCS	E F F	EL F F	BSA
Ewes	15	15	15	15
Ewe lambs	15	15	15	15

6.2.5 One dimension dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

6.2.5.1 Sample preparation

Oocytes were placed on the bench to defrost them at room temperature, and then observed through a binocular microscope in order to have the exact number required for each gel. In each type of media a total of 50 oocytes which have three layers or more of surrounding cells, after removed cumulus by repeated peeping were used per well from both ewes and ewe lambs. Each group of 50 oocytes were placed in a micro centrifuge tube with 0.5 ml PBS, and then centrifuged for 10 mins at 4°C and 13,000 xg. The supernatant was removed and discarded, 200 to 300 µl of sample buffer was added to the precipitate, and then sonicated for 2 seconds, two times each. Again samples were centrifuged as previous described and then the supernatant was removed to a clean centrifuge tube and consequently gel loading. All oocytes processing was done in ice box at 4°C.

6.2.2.1 Running gel

One dimension mini gel (7cm) was done using ewes and ewe lambs oocytes matured in four different media. The gel casting and running as described in section 2.7.1 and 2.7.2, gel staining as described in section 2.7.3.

Statistical analysis

Data from an expansion cytoplasm and cumulus were analyzed by spilt-plot design using GenStat 8 edition and the difference between means were determined using standard error difference and chi-square test was used to evaluate oocytes maturation.

6.3 Results

6.3.1 Oocytes maturation

Ewe lamb oocytes showed similar results to ewe oocytes when matured in TCM-199 with four different additives, when observed after 24 h to reaching MII. There was no difference between age groups. Ewes have more matured oocytes in all types of media compared with ewe lamb oocytes (Table 6.3).

Table.6.3 Number of oocytes that reached MII (%) in each medium for ewes and ewe lambs cultured oocytes cultured in four different media. FCS fecal calf serum, E F F ewe follicular fluid, EL F F ewe lambs follicular fluid, BSA bovine serum albumin.

	FCS	%	E F F	%	EL F F	%	BSA	%
Ewes	12/15	80	11/15	73.3	12/15	80	10/15	66.7
Ewe lambs	11/15	73.3	9/15	60	10/15	66.7	8/15	53.33

6.3.1.1 Cytoplasm diameter measurement

In ewes and ewe lambs, cytoplasm diameter after maturation was significantly ($P<0.05$) greater than before maturation. Overall ewe cytoplasm diameter was significantly ($P<0.05$) greater than ewe lambs cytoplasm diameter before and after maturation in all media (Table 6.4). Mean ewe cytoplasm diameter was 150.9 μm and ewe lamb diameter 114.7 μm ($\text{SED} = 7.13$). In addition, ewe and ewe lamb diameter for oocytes cultured in medium with FCS showed a significantly ($P<0.05$) greater expansion than oocytes cultured in the other media, However, no significant difference was observed between the other types of media.

Table.6.4 Ewes and ewe lambs cytoplasm diameter measurements from 24 h cultured oocytes in four different media. ($\text{SED}=17.72$), FCS: fetal calf serum, E F F: ewe follicular fluid, EL F F: ewe lamb follicular fluid, BSA bovine serum albumin

Media	Ewes			Ewe lambs		
	Before	After	% Change	Before	After	% Change
TCM -199 + FCS	138.8	200	44	105.3	153.3	46
TCM-199 +E F F	138.2	161.7	17	106.8	117.1	9.6
TCM-199 +EL F F	139.8	143	2.3	93.7	117.8	26
TCM-199 + BSA	139	146.3	5.2	105.4	118.4	12.3

6.3.1.2 Cumulus diameter measurement

The results are similar to that observed in section 6.3.1.1. Ewes cumulus diameter expanded more than ewe lambs cumulus. Mean ewes cumulus diameter as 245.2 μm and ewe lambs was 216.3 μm ($\text{SED} = 7.45$) Table 6.5. Moreover, medium with FCS showed a significant ($P<0.05$) difference to other types of media for both ewe and ewe lamb oocytes. In addition, cumulus in both age of groups showed a significant expansion ($P<0.05$) after maturation. However, no difference found on the interaction between age and before and after of cumulus expansion.

Table 6.5 Ewes and ewe lambs 15 cumulus soocyte diameter form each medium were before and after cultured for 24 h in four different media (SED = 32.9), FCS fecal calf serum, E F F

Media	Ewes			Ewe lambs		
	Before	After	% Change	Before	After	% Change
TCM -199 + FCS	246.6	316.2	28	201	261.1	30
TCM-199 +E F F	234.7	260	11	228.9	237.3	8.4
TCM-199 +EL F F	221.7	242	9.2	195.3	214	9.6
TCM-199 + BSA	206.2	236.3	14.6	190.7	202	5.9

ewes follicular fluid, EL F F ewe lambs follicular fluid, BSA bovine serum albumin, fifteen oocytes from each medium per age were used.

6.3.1.3 Cytoplasm total surface area measurements

Overall ewe and ewe lamb cytoplasm total area significantly ($P<0.05$) greater after maturation, mean total surface area for maturation before was $10063 \mu\text{m}^2$ and after maturation $13634 \mu\text{m}^2$ (SED = 1543.7). Moreover, ewe cytoplasm total surface was after maturation was significantly ($P<0.05$) greater than in ewe lamb (Table 6.6). Moreover, FCS had the greater effect than other types media but this effect was not significant.

Table .6.6 Ewe and ewe lamb cytoplasm total surface area, before and after culturing oocytes for 24 h in four different media (SED = 7239.9), FCS fetal calf serum, E F F ewes follicular fluid, EL F F ewe lamb follicular fluid, BSA bovine serum albumin, fifteen oocytes from each medium per age were used.

Media	Ewes			Ewe lambs		
	Before	After	% Change	Before	After	% Change
TCM -199 + FCS	11089	20168	82	9750	14613	50
TCM-199 +E F F	10142	17497	73	8092	12254	41
TCM-199 +EL F F	11328	12619	11.4	10614	10735	1.1
TCM-199 + BSA	11214	17497	56	8152	8783	7.7

6.3.1.4 Cumulus total surface area measurements

The results of this comparison showed, that total surface area of ewe cumulus cells were significantly ($P<0.05$) greater than ewe lamb cumulus cells in all media, mean ewe cumulus total surface area was $20435 \mu\text{m}^2$ and for ewe lambs was $15039 \mu\text{m}^2$ ($\text{SED} = 1135.2$). Moreover, in both ewes and ewe lambs, media with FCS had significantly ($P<0.05$) more effect on cumulus expansion than other media, where no differences were observed between other media. Total surface area in both age groups expanded significantly ($P<0.05$) in all culture media (Table 6.7), the mean surface area before was $14969 \mu\text{m}^2$ and after was $20505 \mu\text{m}^2$ in both ewes and ewe lambs. Ewes and ewe lambs matured oocytes and cumulus expansion (Figure 6.1).

Table 6.7 Ewe and ewe lamb cumulus total surface area, before and after culturing oocytes for 24 h in four different media ($\text{SED} = 6403.6$), FCS fecal calf serum, E F F ewes follicular fluid, EL F F ewe lambs follicular fluid, BSA bovine serum albumin

Media	Ewes			Ewe lambs		
	Before	After	% Change	Before	After	% Change
TCM -199 + FCS	20559	30395	48	11616	19699	70
TCM-199 +E F F	18042	28542	58	11060	15437	40
TCM-199 +EL F F	15767	19151	22	14373	19114	33
TCM-199 + BSA	15173	15850	5	13158	15852	21

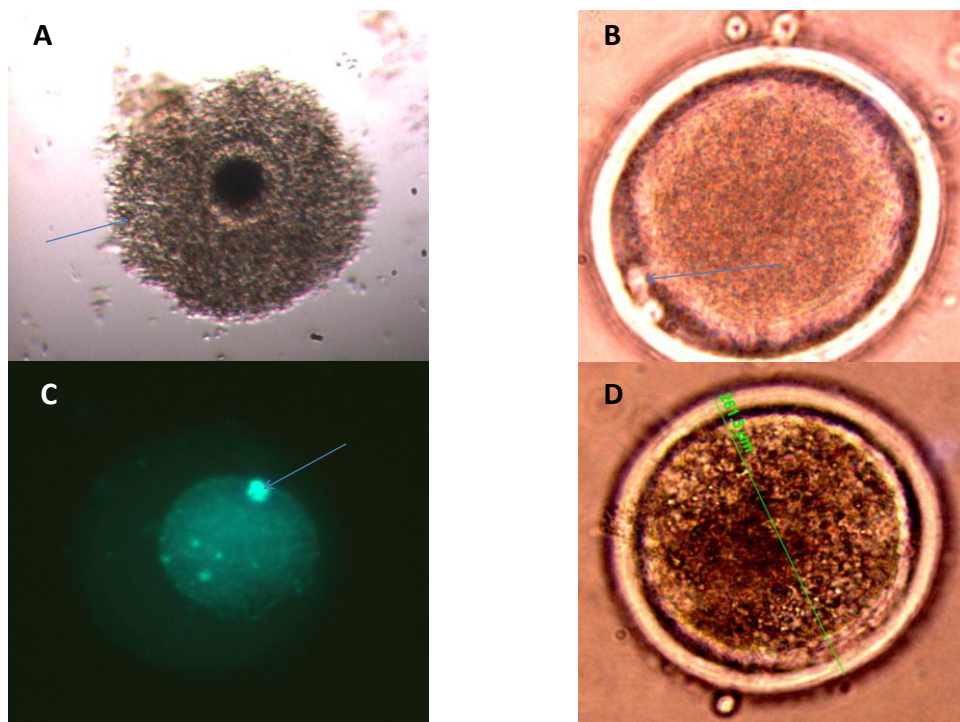


Figure.6.1 Ewe lamb oocyte cultured in TCM-199 supplemented FCS, the arrow shown cumulus expansion (100 x magnification A); and ewe lamb oocyte cultured in TCM-199 supplemented ewe follicular fluid, the arrow shown polar body (400 x magnification B); and ewe oocyte cultured in TCM-199 supplemented FCS, the arrow shown the oocyte (200 x magnification C); and ewe lamb oocyte cultured in TCM-199 supplemented ewe follicular fluid, it shown cytoplasm measuring (400 x magnification D).

6.3.2 Oocytes electrophoresis

6.3.2.1 One dimension dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Ewes and ewe lambs cultured Oocytes in TCM-199 with four different supplementations as following. A) ewe oocytes in FCS. B) ewe oocytes in ewes follicular fluid. C) ewe oocytes in ewe lambs follicular fluid. D) ewe oocytes in BSA. E) ewe lamb oocytes in FCS. F) ewe lamb oocytes in ewes follicular fluid. G) ewe lamb oocytes in ewe lambs follicular fluid. H) ewe lamb oocyte in BSA. I) the ladder (Figure.6.3). The bands are similar in both groups of age, but stronger in ewes than in ewe lambs oocytes.

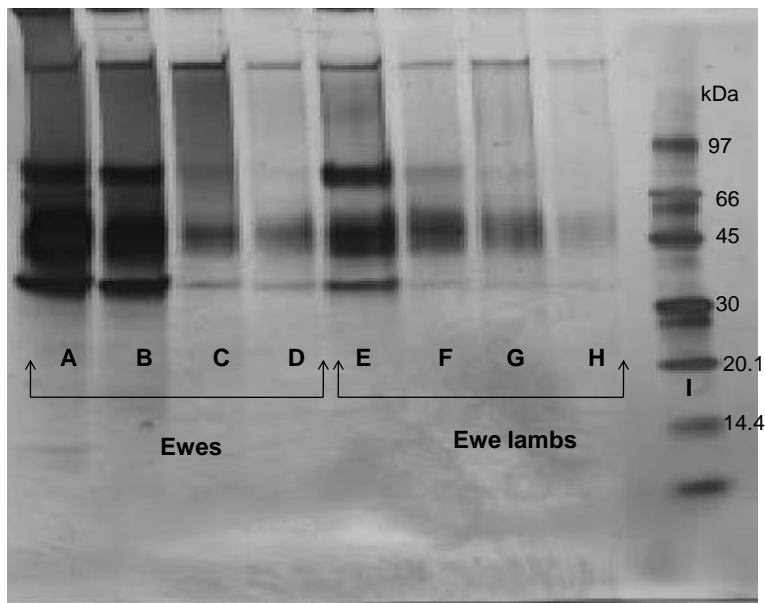


Figure.6.9 Representative patterns of 1 D gel electrophoresis protein from 50 oocytes per well of ewes and ewe lambs matured oocyte A) Ewe oocytes cultured in FCS, B) Ewe oocytes cultured in ewes follicular fluid, C) Ewe oocytes cultured in ewe lambs follicular fluid, D) Ewe oocytes cultured in bovine serum albumen, E) Ewe lambs oocytes cultured in FCS, F) Ewe lambs oocytes cultured in ewes follicular fluid, G) Ewe lambs oocytes cultured in ewe lambs follicular fluid, H) Ewe lambs oocytes cultured in bovine serum albumen, I) The ladder.

6.4 Discussion

Oocyte size of ewe lambs was smaller than that of ewe oocytes, even though both are capable to achieve a successful development to MII. These results agree with Ledda *et al.* (1999 a and b) who reported that in juvenile ewes a high percentage of oocytes from small follicles are able to achieve full meiotic progression. However, these results are in contrast with other studies which demonstrated that oocytes from juvenile and adult have different developmental capability for *in vitro* development (Armstrong, 2001, O'Brien *et al.*, 1997).

The tendency for fewer oocyte to development MII may be related to the smaller oocytes of ewe lambs, which were harvested from smaller size follicles. Overall the rate of nuclear maturation was similar between ewe lambs and ewes, therefore mature competence was unaffected in ewe lambs, which agrees with Armstrong (2001) where nuclear maturation was normal but not in maturation cytoplasm. Cytoplasm/cumulus expansion is greater in FCS media, as it contains hormones, growth factors and proteins which are needed for maturation. Surface area increases tended to be greater in ewes follicular fluid than ewe lambs follicular fluid. It can be suggested that ewes follicular fluid contains more factors required for maturation. Moreover, ewe cytoplasm and cumulus were expanded to a greater extent than those in ewe lambs, as follicle size can influence the quality of the oocyte (Sirard *et al.*, 2006). These results agrees with Gandolfi *et al.*(1998), who found the same difference between oocytes recovered from calf and adult cow. This could explain the difference in the capability of the ewe lamb oocytes to reach MII during maturation. Full oocyte maturation is not necessarily indicated by the MII stage but rather by the end of several processes including cytoplasmic organelle distribution and protein synthesis (Moor and Crosby, 1986, Thibault *et al.*, 1987).

Basically when oocytes were cultured in different media contains FCS, ewe follicular fluid, ewe lamb follicular fluid or BSA, there was no significant difference in oocytes maturation and cumulus expansion area. This may have been result of different level of protein in the media. Overall, protein amount was greater in FCS than in ewe follicular fluid. Protein was also higher in ewe follicular fluid than in ewe lambs follicular fluid which in turn was found to be a greater amount than in BSA in both ewes and ewe lambs, which agrees with the diameter surface area measurements.

The result from 1D electrophoresis shows the similar bands and the same MW marker in both ewe and ewe lamb gels, but in ewe the bands were denser than in the ewe lambs. This agrees with Gandolfi *et al.*(1998), who reported that oocytes recovered from calf ovaries, on average, differ in diameter and protein synthesis than in cows. Protein synthesis was higher in ewe and ewe lamb oocytes cultured with FCS than other media. The largest bands were estimated between 30 to 97 kDa and were approximately similar in both ages with different media. The differences between ewes and ewe lamb band in density after maturation, suggest maturation was greater in oocytes and cumulus in ewes compared to ewe lamb. Ewe Cytoplasm diameter was 150.9 μm compared with ewe lambs 114.7 μm . This could be an important parameter that affects ewe lamb oocytes development competence. Also this could be due to abnormal and low oocytes incomplete development competence in ewe lambs. This agrees with an *in vivo* study with juvenile ewes and adult ewes by Quirke and Hanrahan (1977), who reported that there was reduced development competence of juvenile sheep oocytes compared with adult ewes. In addition to the lowest development of ewe lamb oocytes, probably due to *in vivo* ovarian response to gonadotrophin hormones, which might cause more matured follicles in ewes compared with ewe lambs and consequently more matured ewe oocytes than in the ewe lamb. Also oocytes from ewe lamb, come from small follicles compared with ewe follicles and therefore, were the differences in protein synthesis between the two age groups may play a role in the control of meiotic resumption. Protein syntheses are necessary for the conversion from MI to MII and for extrusion of the first polar body (Sirard *et al.*, 1989). In general media with high protein give a high expansion in diameter.

The results of this study differ from Armstrong (2001), who reported that in several mammalian species, oocytes from prepubertal animals have limited potential to undergo normal embryogenesis and produce viable offspring. Ledda *et al.* (1997) summarised that there was lower development

competence for prepubertal lamb oocytes than ewe oocytes when matured in the absence of added hormones *in vitro*. Another study found that lamb and calf oocytes did not significantly differ, whether matured *in vivo* or *in vitro*, and the rates of development to blastocyst stages in culture were similar to those observed in embryos derived from adult donors (Armstrong *et al.*, 1997).

Adding LH and FSH to the media enhanced developmental competence of oocytes by activating biosynthetic processes in the oocyte. Moreover, there was a greater response of ewe oocytes and cumulus to maturation than ewe lamb oocytes, which revealed more FSH receptors in ewe oocytes and therefore more FSH binding sites in ewe oocytes compared with a low response in lamb oocytes. Furthermore, FSH is used to increase blastocyst yield and to obtain oocytes to undergo meiotic maturation. Adding LH was to induce oocyte maturation.

6.5 Conclusion

Ewe lamb oocytes were smaller than ewe oocytes and have less development competence than adult oocytes. Nuclear maturation in ewe lambs was similar to that in adult ewes. Medium with FCS enhanced a significant cytoplasm/cumulus expansion among the media maturation used in this experiment. In addition, 1D electrophoresis showed similar bands with the same MW kDa, with more protein in ewe oocytes compared with ewe lamb oocytes (Moor and Crosby, 1986, Thibault *et al.*, 1987).

Chapter 7

General Discussion

Subfertility has long been recognized as the main factor affecting productivity in ewe lambs (Dyrmundsson, 1973a). Moreover, ewe lambs are less responsive to FSH/LH compared with adults *in vivo* (Khan *et al.*, 2007). Most experiments have been conducted using *in vivo* techniques in order to improve ewe lamb reproductive function. These experiments however, were unable to improve reproductive performance. Using *in vitro* studies will add to our understanding and help to contribute to the improvement of ewe lambs reproductive function.

Basal oestradiol concentration was similar in media from ewe and ewe lamb cultured follicles but not in follicular fluid. Culture medium with FCS supplementation improved oestradiol secretion in both age groups, but more so in ewes than in ewe lambs which may indicate that more hormone receptors are present in ewe follicles than in those of ewe lambs as FCS contains growth factors, proteins and hormones. In both ewe and ewe lamb cultured follicles, oestradiol was secreted into the medium throughout the 8 hour incubation period, which indicated that the tissue was still living and producing hormone, but hormone production decreased with incubation time, suggesting that the tissue was becoming atretic. Perhaps a culture system in which the follicles were exposed to a continuous flow of media would have given more physiological results. More work requires, using more frequent incubation times (one hour interval) for more than 24 h. This will determine the optimum time for hormone secretion, and also the point where hormone is no longer secreted.

Media supplemented with FSH and LH or hCG increased oestradiol secreted in the media of ewe and ewe lamb follicles and oestradiol concentration was increased with increased follicle size in both age groups. FSH treatment *in vivo* has been shown to increase oestradiol concentration (Campbell *et al.*, 1995a, Valasi *et al.*, 2007). Oestradiol secretion was higher in

ewes than in ewe lambs. Which may be explained by a greater number of cells in the theca and granulosa layers and/or an increased number of hormone receptors in ewe theca and granulosa layers. This would lead to increased enzyme activity and the amount of cholesterol conversion into oestradiol (Beck *et al.*, 2003). However, hormone secretion was declined after 2 h of incubation which indicated that cultured follicular tissue may experience atresia. Further work is required measuring the enzyme activity in ewe and ewe lamb theca and granulosa layers. Using *in vitro* tissue culture and then enzyme extraction sequentially to determine whether both ewes and ewe lambs have the same enzymatic activity and whether it is related to hormone secretion.

FSH/LH and hCG stimulated more progesterone secretion in ewe follicles than in ewe lamb follicles when used at different concentrations for several incubation times. More cholesterol was converted into progesterone in ewe theca cells which could be due to the CYP17 and 3 β HSD enzymes which govern cholesterol conversion to progesterone being more active in ewes than in ewe lambs. In addition, there may have been more LH receptors in ewe follicles, therefore, more progesterone would have been synthesised in ewe follicles than in ewe lamb follicles. In addition, FCS supplementation to the culture medium also increased progesterone and oestradiol secretion and stimulation with hormones or FCS gave a more accurate reflection of the difference in steroidogenic capacity between age groups. In future work, slices could be taken from ewe and ewe lamb follicles shell in sequence to count FSHR and LHR, this would determine whether there is any difference in the number of these receptors between ewes and ewe lambs and, thus the different amounts of hormone secreted from these follicles.

The results for the follicles from animals given *in vivo* hormone treatment were similar to those treated *in vitro*. The oestradiol secretion into the medium after different times of incubation was similar for ewe and ewe lamb follicles when treated by exogenous ovagen. This treatment has the same affect as oFSH on follicles *in vitro* in both age groups and a similar response of follicles to the treatment. Furthermore, no difference in progesterone concentration was found between medium and large follicles in either age group. Also similar amounts of hormone were secreted from ewes and ewe lambs. This may be attributable to similar enzyme activity in the theca and granulosa cells which are involved in steroidogenesis. Treatment *in vivo* with hCG caused a significant increase in ewe oestradiol secreted compared with ewe lambs. This suggests that ewe follicles may have more LH receptors than those in ewe lamb follicles. Which means more binding sites for hCG result in more enzyme activity, to secrete more hormone, which resulted in more oestradiol secreted into the medium, which is in agreement with Driancourt *et al.*, (2001b) who found that follicular oestradiol secretion increased with age in cattle. Oestradiol secretion decreased with increased incubation time which indicates that the tissue may become atretic.

The increased progesterone production in ewe follicles following ovagen treatment could be due to increased activity of enzymes in theca cells, in addition, ewe follicles may be more luteinized than ewe lamb follicles. Also ewe lambs require more cholesterol due to growth and metabolism, therefore less cholesterol is available to be converted into progesterone. The increase in media progesterone levels with increased follicle size was possibly due to the increased numbers of cells in the theca layer and therefore more cholesterol conversion into progesterone, which agrees with Beck *et al*, (2003). In addition it possible the cells number in the theca layer of ewes were more than those in ewe lambs and more matured to produce more

progesterone than in ewe lamb follicles. Also ewe follicles more luteinization may have occurred than in ewe lambs.

The same results were found for progesterone concentrations between ewes and ewe lambs treated with ovagen plus hCG, where higher concentrations of oestradiol and progesterone were found in the media of ewe follicles than in ewe lambs. Overall in both age groups hormones level were higher in groups treated with ovagen than in those treated with ovagen plus hCG. In groups treated by ovagen plus hCG, this could be explained by the amounts of cholesterol converted into progesterone and then into oestradiol being higher than that in ovagen treated groups and higher in ewes compared with ewe lambs.

Plasma progesterone concentration after CIDR treatment increased in both treatments and age groups. It also increased more over time in ewes than in ewe lambs. The different response between age groups for the CIDR could be due to the vaginal surface area, which is larger in ewes than in ewe lambs and consequently, the amount of progesterone absorbed into the blood system would be greater in ewes than in ewe lambs. Furthermore, metabolic rate is faster in ewe lambs and therefore hormone clearance rates may be faster compared with adult ewes.

In vitro CL function was lower in ewe lambs compared with ewes, even though there was no difference between luteal tissue weights. This concurs with *in vivo* results by both Downing (1980), who reported that progesterone concentrations of 1-3 ng/ml in ewe lambs compared with 3.6-5.4 ng/ml in ewes during early gestation and Khan *et al.* (2007), who reported that, luteal function during early pregnancy is significantly higher in ewes than in ewe lambs. The lower amount of progesterone in ewe lambs could be one of the reasons for ewe lamb

subfertility. Moreover, this may be due to a greater number of small luteal cells in ewe luteal tissue which contain more LH receptors and therefore produce more progesterone than ewe lamb luteal tissue. Furthermore, ewe CL may include a greater number of luteal cells than ewe lamb CL tissue. Further work is required to investigate the differences between both ages. This could be done by counting the number of small and large luteal cells in both age groups using tissue slices. In addition these cells could be cultured in different media to determine the amount of progesterone secretion.

It has been reported that *in vivo* luteal function in ewes is greater than in ewe lambs (Khan *et al.*, 2007, Khan *et al.*, 2006). However, the decreased progesterone concentration in ewe luteal tissue compared with that of ewe lambs, when treated with LH, could be due to more responsive of small luteal cells in ewe lamb CL to LH moreover, the ewe CL tissue could be undergoing atresia consequently decreased the amount of progesterone secreted. However, the increased progesterone concentrations in ewe luteal tissue when treated with hCG compared with that in ewe lambs may indicate that ewe luteal tissue is more sensitive to hCG than ewe lamb tissue. Moreover, it could indicate inadequate ewe lamb luteal function as compared with ewes. Progesterone secretion into the media declined after 2 h with increased incubation time and this result is in contrast with Bramley *et al.*(2005), who reported that progesterone concentrations were increased with increased incubation time up to 4 h.

Protein electrophoresis for follicular shell tissue and CL tissue and gels from follicles cultured in FSH/LH support the results for the difference in hormone secretion between ewes and ewe lambs. In both treated and untreated follicles and for different follicular shell sizes ewe gels contained more spots than ewe lamb gels. The number of protein spots increased with

increased follicle size and with FSH and LH treatment. Moreover, more abundant protein spots were estimated between MW of 30 to 100 kDa. This increase might be due to ewe follicles being more responsive to FSH and LH, which results in more activated oestrogenic enzymes than those in ewe lambs.

The results for electrophoresis showed more protein spots in ewe average gels than in ewe lamb average gels in groups treated with ovagen or ovagen and hCG. The majority of large spots were estimated between 30 to 45 kDa, which suggests that the protein in this range of MW might have a positive relation with steroidogenic hormone synthesis and may be related to the increase in hormone secretion *in vitro*. Overall the increase protein spots in ewes compared with ewe lamb gels agree with the results for hormones analysis, indicating that the greater response of ewe follicles for hormone stimulation was due to the increased activity of enzymes governing steroidogenesis. In the average gels results were eliminated of the dust spots, but there were not removed from not from singles gels and may therefore have affected results in chapter 4.

Moreover, up regulated spots and unique spots were more abundant in ewe gels compared with ewe lamb gels. Future work can be carried out in electrophoresis investigation, such as detection of up regulated and unique spots that are present in one age-group gels than the other. This can be done using mass spectrometry and western blotting to identify the unique spots. This will help to identify which type of proteins are present in each spot and whether there is any relation of these proteins to the enzymes that govern steroidogenesis. In addition, targeted antibodies could be used to look at changes *in vivo* and may be a good method to determine the differences between the two ages.

The result from oocyte maturation revealed that oocytes obtained from ewe lambs were smaller than those obtained from adult ewes, which agrees with Ledda *et al.*(1999 a, b) and nuclear maturation was similar to that for adult oocytes, which agrees with Armstrong, (2001). Even though the ewe lamb oocytes are smaller than from ewe oocytes, these oocytes successfully reached MII. Using TCM-199 with FCS significantly enhanced cytoplasmic and cumulus expansion compared with other types of media. This expansion was greater in ewe oocytes than in those of ewe lambs as it in cattle, where the expansion was greater in cow oocytes compared with calf oocytes (Gandolfi *et al.*, 1998). Similar bands estimated between 30 and 97 kDa were observed in the 1D gel for ewe and ewe lamb oocytes, but with denser bands in ewes compared with ewe lambs. The lower competence of ewe lamb oocytes may be due to ewe lamb ovaries being less sensitive to pituitary hormones which may cause fewer oocytes to mature. In this experiment, the adding of FSH and LH did not seem to improve oocyte maturation. In addition, culture in ewe lamb follicular fluid resulted in less oocyte development than that in ewe follicular fluid, but more oocytes developed in lamb follicular fluid than for those cultured in medium supplied with BSA. This suggests that ewe lamb follicle fluid contains less hormones and protein compared with ewe follicle fluid and this result in slower oocyte maturation. In the future, proteins contained in each age of oocytes should be identified, through using radio labelling in oocytes from each age groups. It should be possible to determine the proteins present and to find out the difference between these proteins. It could also be found by measuring hormones activity and using western blotting to identify the special proteins present in one age group of oocytes compared with the other.

7.1 Conclusions

Basal oestradiol and progesterone secretion were similar for follicles cultured *in vitro* from ewes and ewe lambs. The hormone secretion was increased with increasing follicle size. However, follicles from ewes cultured *in vitro* with FCS or gonadotrophins produced more oestradiol than follicles from ewe lambs. Progesterone secretion from corpora lutea cultured *in vitro* was greater for ewes than for ewe lambs. Proteomic analysis of follicular and luteal tissue indicated ewe tissue contained more protein spots than that for ewe lambs. *In vitro* gonadotrophin stimulates more oestradiol secretion in ewes and ewe lambs, but showed more progesterone secretion in ewe lamb follicles compared with ewes. That could be due to FSHR and LHR, which were less in ewe follicles compared with ewe lamb follicles. For that reason more hormone binding sites were localized in ewe lambs compared with ewes. However, oestradiol and progesterone concentration in ewe follicles cultured TCM-199 plus hCG were more than those of ewe lambs. Ewe lamb CL tissue produced less progesterone than ewe tissue when cultured different media. Proteomic analysis indicated that gels from follicle shells cultured in TCM-199 plus FSH and LH have a greater effect on protein spots compared with control and more so in ewe gels than in ewe lamb gels.

Follicles cultured from ewes and ewe lambs after *in vivo* ovagen (FSH) treatment produced a similar amount of oestradiol and progesterone. Follicles cultured from ewes given FSH and hCG *in vivo* secreted more oestradiol than those from ewe lambs given an identical treatment. Proteomic analysis of follicles collected from ewes and ewe lambs given either FSH or FSH plus hCG *in vivo* indicated increased protein up-regulation and spot concentration in ewes compared to ewe lambs. Oocytes collected from ewe ovaries and matured *in vitro* were larger and showed greater nuclear and cytoplasmic maturation and cumulus cell expansion than those

collected from ewe lambs. Furthermore, 1D analysis suggested that protein band concentrations were greater in ewes than in ewe lambs.

However, there are different factors that have to be studied in order to illustrate the main difference between ewes and ewe lambs *in vitro* which help until to improve ewe lamb reproduction function. Therefore, future investigation should consider using more animals for each treatment, using prepubertal, pubertal and adult ewes. In addition, CL tissue should be cultured for 2 h. Using 2D electrophoresis for frozen oocytes and amino acid labelling can assist protein identification and also western plot could be used to identify types of protein.

References

- ABECIA, F. & ZUNGA, O. (2001) Differences in reproductive performance, embryo development, interferon-tau secretion by the conceptus and luteal function in ewe lambs synchronized in oestrus before or after the spontaneous onset of luteal activity preceding puberty. *Reprod Domest Anim*, 36, 73-77.
- ABECIA, J. A., VALARES, J. A., FORCADA, F., PALACIN, I., MARTIN, S. & MARTINO, A. (2007) The effect of melatonin on the reproductive performance of three sheep breeds in Spain. *Small Ruminant Research*, 69, 10-16.
- ABRAMOVITZ, M., BOIE, Y., NGUYEN, T., RUSHMORE, M. A., METTERS, K. M., SLIPETZ, T. M. & GRYGORCZYK. (1994) Cloning and expression of a cDNA for the humanoid FP receptor. *J Biol Chem*, 269, 1356-1360.
- ACCARDO, C., DATTENA, M., PILICHI, S., MARA, L., CHESSA, B. & CAPPAL, P. (2004) Effect of recombinant human FSH and LH on in vitro maturation of sheep oocytes; embryo development and viability. *Anim Reprod Sci*, 81, 77-86.
- ACOSTA, T. J., HAYASHI, K. G., OHTANI, M. & MIYAMOTO, A. (2003) Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. *Reproduction*, 125, 759-767.
- ACRITOPOULOU, S. & HARESIGN, W. (1980) Response of ewes to single injection of analogue of PGF₂ given at different stages of oestrous cycle. *J. Reprod. Fertil.*, 58, 219-223.
- ADAM, C. L. & FINDLAY, P. A. (1997) Effect of nutrition on testicular growth and plasma concentrations of gonadotrophins, testosterone and insulin-like growth factor I (IGF-I) in pubertal male Soay sheep. *Reproduction and Fertility*, 111, 121-125.
- ADAMS, G. P., EVANS, A. C. & RAWLINGS, N. C. (1994) Follicular waves and circulating gonadotrophins in 8-month-old prepubertal heifers. *J Rproduction and Fertilit*, 102, 463-470.
- ADASHI, E. Y., RESNICK C, E., D'ERCOLE, A. J., SVOBODA, M. E. & VAN WYK, J. J. (1985) Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocr Rev.*, 6, 400-420.
- ADASHI, E. Y., RESNICK, C. E. & ROSENFELD, R. G. (1990) Insulin-Like Growth Factor-I (Igf-I) And Igf-Ii Hormonal Action In Cultured Rat Granulosa-Cells - Mediation Via Type-I But Not Type-Ii Igf Receptors. *Endocrinology*, 126, 216-222.
- AGRAWAL, K. P., SHARMA, T., SEXENA, C AND SHARMA, N. (1995) Chronology of first meiotic events of caprine oocytes matuted *in vitro* Ind. *Animal Science*, 65, 285-288.
- AL-GUBORY, K. H., HERVIEU, J. & FOWLER, P. A. (2003) Effects of pregnancy on pulsatile secretion of LH and gonadotrophin-releasing hormone-induced LH release in sheep: a longitudinal study. *Reproduction*, 125, 347-355.
- ALI, A. & SIRARD, M. A. (2002) Effect of the absence or presence of various protein supplements on further development of bovine oocytes during in vitro maturation. *Biology Of Reproduction*, 66, 901-905.
- ALLISON, A. J. & ROBINSON, T. J. (1970) Effect Of Dose Level Of Intravaginal Progestagen On Sperm Transport, Fertilization And Lambing In Cyclic Merino Ewe. *Journal Of Reproduction And Fertility*, 22, 515-520.
- ANDERSON, L. L., NEOL, F. C. & MELAMPY, R. M. (1961) Hysterectomy and ovarian function in beef heiferes. *American Journal of Vet. Res.*, 23, 794- 802.

- ANDERSON, L. L., RATHMACHER, R. P., AND MELAMPY, R. M. (1966) The uterus and unilateral regression of corpora lutea in the pig. *American Journal of Physiology*, 210, 611-614.
- ANDRIESZ, C. & TROUNSON, A. (1995) The effect of testosterone on the maturation and development capacity of murine oocytes *in vitro* *Hum Reprod*, 10, 2377-2381.
- ARENDT, J., SYMONS, A. M., LAUD, C. A. & PRYDE, S. J. (1983) Melatonin Can Induce Early Onset Of The Breeding-Season In Ewes. *Journal Of Endocrinology*, 97, 395-400.
- ARMSTRONG, D. G., BAXTER, G., GUTIERREZ, C. G., HOGG, C. O., GLAZYRIN, A. L., CAMPBELL, B. K., BRAMLEY, T. A. & WEBB, R. (1998) Insulin-like growth factor binding protein -2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: Effect of gonadotropins and developmental status. *Endocrinology*, 139, 2146-2154.
- ARMSTRONG, D. G., GUTIERREZ, C. G., BAXTER, G., GLAZYRIN, A. L., MANN, G. E., WOAD, K. J., HOGG, C. O. & WEBB, R. (2000) Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. *Journal Of Endocrinology*, 165, 101-113.
- ARMSTRONG, D. G. & WEBB, R. (1997) Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. *Rev Reprod*, 2, 139-46.
- ARMSTRONG, D. T. (2001) Effects of maternal age on oocyte developmental competence. *Theriogenology*, 55, 1303-1322.
- ARMSTRONG, D. T. & GRINWICH, D. L. (1972) Blockade of spontaneous and LH-induced ovulation in rats by indomethacin, an inhibition of prostaglandin biosynthesis. *Prostaglandins*, 1, 21-36.
- ARMSTRONG, D. T., HOLM, P., IRVINE, B., PETERSEN, B. A., STUBBINGS, R. B., MCLEAN, D., STEVENS, G. & SEAMARK, R. F. (1992) Pregnancies And Live Birth From Invitro Fertilization Of Calf Oocytes Collected By Laparoscopic Follicular Aspiration. *Theriogenology*, 38, 667-678.
- ARMSTRONG, D. T., KOTARAS, P. J. & EARL, C. R. (1997) Advances in production of embryos in vitro from juvenile and prepubertal oocytes from the calf and lamb. *Reproduction Fertility and Development*, 9, 333-339.
- ARMSTRONG, D. T., KOTARAS, P. J. & EARL, C. R. (1997a) Advances in production of embryos in vitro from juvenile and prepubertal oocytes from the calf and lamb. *Reproduction. Fertility and Development* 9, 333-339.
- ATTARDI, B., KLATT, B., HOFFMAN, G. E. & SMITH, M. S. (1997) Facilitation or inhibition of the estradiol-induced gonadotropin surge in the immature rat by progesterone: Regulation of GnRH and LH messenger RNAs and activation of GnRH neurons. *Journal Of Neuroendocrinology*, 9, 589-599.
- AVERY, B., STROBECH, L., JACOBSEN, T., BOGH, I. B. & GREVE, T. (2003) In vitro maturation of bovine cumulus-oocyte complexes in undiluted FF:effect on nuclear maturation, pronucleus formation and embryo development *Theriogenology*, 59, 987-999.
- BACHAROVA, R. (1985) Gene expression during oogenesis and oocyte development in mammals. IN BROWDER, L. W. (Ed.) *Developmental Biology: A Comprehensive Synthesis*. New York, Plenum.
- BAIRD, D. T. (1978) Pulsatile Secretion of LH and Ovarian Estradiol During the Follicular Phase of the Sheep Estrous Cycle.

- BAIRD, D. T., CAMPBELL, B. K., MANN, G. E. & MCNEILLY, A. S. (1991) Inhibin and estradiol In the control of Fsh-secretion In the sheep. *Journal Of Reproduction And Fertility, Supplement*, 43, 125-138.
- BAIRD, D. T. & MCNEILLY, A. S. (1981) Gonadotrophic control of follicular development and function during the oestrous cycle of the ewe. *J Reprod Fertil Suppl*, 30, 119-133.
- BAIRD, D. T. & S., M. A. (1981.) Gonadotrophic control of follicular development and function during the oestrous cycle of the ewe. *J.Reprod. Fertil. Supplement* 30., 119-133.
- BAIRD, D. T. & SMITH, K. B. (1993) Inhibin and related peptides in the regulation of reproduction. . In *Oxford Review of reproductive biology*, 15, 191-232.
- BAIRD, D. T., SWANSTON, I. & SCARAMUZZI, R. J. (1976) Pulsatile Release Of Lh And Secretion Of Ovarian Steroids In Sheep During Luteal Phase Of Estrous-Cycle. *Endocrinology*, 98, 1490-1496.
- BAKER, T. G. (1963) A quantitative and cytological study of germ cells in human ovaries. *Proceedings of the Royal Society of London Series B - Biological Sciences*, 158, 417-433.
- BARNES, F. L. & FIRST, N. L. (1991) Embryonic transcription in vitro cultured bovine embryos. *Mol Reprod Dev*, 29, 117-123.
- BARRETT, J., BROPHY, P. M. & HAMILTON, J. V. (2005) Analysing proteomic data. *International Journal for Parasitology*, 35, 543-553.
- BARTLEWSKI, P., BEARD, A., COOK, S., CHANDOLIA, R., HONARAMOOZ, A. AND RAWLINGS, N. (1999b) Ovarian antral follicular dynamics and their relationships with endocrine variables through the oestrous cycle in breeds of sheep differing in prolificacy. . *J. Reprod. Fertil.*, 115, 111-124.
- BARTLEWSKI, P. M., BEARD, A. P., COOK, S. J. & RAWLINGS, N. C. (2002) Ovarian activity during sexual maturation and following introduction of the ram to ewe lambs. *Small Ruminant Research*, 43, 37-44.
- BARTLEWSKI, P. M., BEARD, A. P. & RAWLINGS, N. C. (2000c) An ultrasound-aided study of temporal relationships between the patterns of LH/FSH secretion, development of ovulatory-sized antral follicles and formation of corpora lutea in ewes. *Theriogenology*, 54, 229-245.
- BARTLEWSKI, P. M., BEARD, A. P. & RAWLINGS, N. C. (2006) Ultrasonographic study of antral follicle development during sexual maturation in ewe lambs. *Small Ruminant Research*, 63, 189-198.
- BATHAEI, S. (1996) Breeding season and oestrous activity of Iranian fat-tailed Mehraban ewes and ewe lambs. *Small Ruminant Research*, 22, 13-23.
- BAULIEU, E. E. & KELLY, P. A. (1990) In: *Hormones Chapman and Hall*. New York, London
- BAVISTER, B. D., ROSEHELLEKANT, T. A. & PINYOPUMMINTR, T. (1992) Development Of In Vitro Matured In Vitro Fertilized Bovine Embryos Into Morulae And Blastocysts In Defined Culture Media. *Theriogenology*, 37, 127-146.
- BECK, N. F. G. & DAVIES, M. C. G. (1994a) The effect of stage of breeding season or pre-mating oestrogen and progesterone therapy of fertility in ewe lambs. *J. Anim. prod.*, 59, 429-434.
- BECK, N. F. G., DAVIES, M. C. G. & DAVIES, B. (1996b) A comparison of ovulation rate and late embryonic mortality in ewe lambs and ewes and the role of late embryo loss in ewe lamb subfertility. *Animal Science*, 62, 79-83.

- BECK, N. F. G., JONES, M., DAVIES, B., MANN, G. E. & PETERS, A. R. (1996a) The effect of GnRH analogue (buserelin) treatment on day 12 post mating on ovarian structure and plasma progesterone and oestradiol concentration in ewes. *Animal Science*, 63, 407-412.
- BECK, N. F. G., KHALID, M., CHARLES, J. M., ABBAS, S. K. & CARE, A. D. (2003) Relationship between intrafollicular concentrations of parathyroid hormone-related peptide (PTHrP) and steroid hormones in oestrogenic and non-oestrogenic ovarian follicles in the mare. *Animal Reproduction Science*, 76, 91-97.
- BECK, N. F. G., PETERS, A. R. & WILLIAMS, S. P. (1994b) The Effect Of GnRh Agonist (Buserelin) Treatment On Day 12 Post Mating On The Reproductive-Performance Of Ewes. *Animal Production*, 58, 243-247.
- BEHRMAN, H. R., MACDONALD, G. J. & GREEP, R. O. (1971) Regulation of ovarian cholesterol esters: evidence for the enzymatic sites of prostaglandin -induced loss of corpus luteum function. *Lipids*, 6, 791-796.
- BEKER, A. R. C. L., COLENBRANDER, B. & BEVERS, M. M. (2002) Effect of 17[beta]-estradiol on the in vitro maturation of bovine oocytes. *Theriogenology*, 58, 1663-1673.
- BERISHA, B., MEYER H. & SHAMS, D. (2004) the expression of fibroblast growth factor (FGF) family members in bovine follicles during peri-ovulatory phase. *Vet Med Austria/Wiener Tierarztlche Monatsschrift*, 91 (Suppl. 2), 10-11.
- BERISHA, B. & SCHAMS, D. (2005) Ovarian function in ruminants. *Domestic Animal Endocrinology*, 29, 305-317.
- BERISHA, B., SCHAMS, D., KOSMANN, M., AMSELGRUBER, W. & EINSPANIER, R. (2000) Expression and localization of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2) during the final growth of bovine ovarian follicles. *J. Endocrinol*, 167, 371-382.
- BERRIDGE, M. J. & IRVINE, R. F. (1984) Inositol triphosphate: a novel second messenger signal transduction. *Nature*, 312, 315-321.
- BESNARD, N., PISSELET, C., MONNIAUX, D., LOCATELLI, A., BENNE, F., GASSER, F., HATEY, F. & MONGET, P. (1996) Expression of messenger ribonucleic acids of insulin-like growth factor binding proteins-2, -4, and -5 in the ovine ovary: Localization and changes during growth and atresia of antral follicles. *Biology Of Reproduction*, 55, 1356-1367.
- BILLIG, H., CHUN, S. Y., EISENHAEUER, K. & HSUEH, A. J. W. (1996) Gonadal cell apoptosis: Hormone-regulated cell demise. *Human Reproduction Update*, 2, 103-117.
- BISTER, J. L., NOEL, B., PERRAD, B., MANDIKI, S. N., MBAYAHAGA, J. & PAQUAY, R. (1999) Control of ovarian follicles activity in the ewe. *Domest Anim Endocrinol*, 17, 315-28.
- BITTMAN, E. L., KARSCH, F. J. & HOPKINS, J. W. (1983) Role Of The Pineal-Gland In Ovine Photoperiodism - Regulation Of Seasonal Breeding And Negative Feedback Effects Of Estradiol Upon Luteinizing-Hormone Secretion. *Endocrinology*, 113, 329-336.
- BJERSING, L., HY, M. F., KANN, G., MOOR, R. M., NAFTOLIN, F., SCARAMUZZI, R. J., SHORT, R. V. & YOUNGLAI, E. V. (1972) Changes in gonadotrophins, ovarian steroids and follicular morphology in sheep at oestrus. *J Endocrinol*, 52, 465-79.
- BOLAND, M. P., GORDON, I. R. & L., K. D. (1978) The effect of treatment by prostaglandin analogue (ICI-80996) or progestagen (SC-9880) on ovulation and fertilization in cyclic ewes. *Journal Agriculture Science* 91, 727-730.

- BOROWCZYK, E., JOHNSON, M. L., BILSKI, J. J., BILSKA, M. A., REDMER, D. A., REYNOLDS, L. P. & GRAZUL-BILSKA, A. T. (2007) Role of gap junctions in regulation of progesterone secretion by ovine luteal cells in vitro. *Reproduction*, 133, 641-651.
- BORWICK, S. C., RHIND, S. M. & MCMILLAN, S. R. (1994) Ovarian steroidogenesis and development in foetal Scottish Blackface ewes, undernourished in utero from conception. *Journal of Reproduction and Fertility*, Abst. Series 14, 14.
- BORWICK, S. C., RHIND, S. M. & MCMILLAN, S. R. (1995) Effects of undernutrition from the time of mating and ovarian development in foetal sheep at 62d gestation. *Journal of Reproduction and Fertility*, Abst. Series 15, 52.
- BOSHOF, D. A., BURGER, F. J. L. & CRONJE, J. A. (1975) Sexual activity of Romanov-Karakul crosses under extensive condition *South African Journal of Animal Science*, 5, 91.
- BOWSTEAD, J. E. (1930) The effect of breeding immature ewes. *Scienc Agriculture*, 10, 429.
- BRADEN, T. D., SAWYER, H. R. & NISWENDER, G. D. (1989) Functional and morphological characteristics of the first corpus luteum formed after parturition in ewes.
- BRADEN, T. D., SAWYER, H. R. AND NISWENDER, G. D. (1989) Functional and morphological characteristics of the first corpus luteum formed after parturition in ewes. *J.Reprod. Fertil*, 86, 525-533. [Abstract].
- BRAMLEY, T. A., STIRLING, D., MENZIES, G. S. & BAIRD, D. T. (2004) Reduced LH sensitivity in vivo and in vitro of corpora lutea induced during anoestrus by GnRH, and during the late breeding season, in Scottish Blackface ewes. 183, 517-526.
- BRAMLEY, T. A., STIRLING, D., MENZIES, G. S. & BAIRD, D. T. (2005) Corpora lutea induced by gonadotrophin- releasing hormone treatment of anoestrus Walsh Mountain ewes: reduced sensitivity to luteinizing hormone *in vivo* and to chronoc gonadotrophin *in vitro*. . *Reproduction*, 129, 61-73.
- BRAND, A. & JONG, W. (1973) Qualitative And Quantitative Micromorphological Investigations Of Tertiary Follicle Population During Estrous-Cycle In Sheep. *Journal Of Reproduction And Fertility*, 33, 431-&.
- BRIGGS, H. M. (1936) Some effects of breeding ewe lambs. *North Dakota Sta Bull*, 285.
- BYSKOV, A. G. (1978) Follicular Atresia. IN JONES, R. (Ed.) *The Vertebrate Ovary*. New York, Plenum.
- BYSKOV, A. G. (1979) IN MIDGLEY, A. R. & SADLER, W. A. (Eds.) *Ovarian Follicular Development and Function*. New York, Raven Press.
- BYSKOV, A. G. (1986) Differentiation of the mammalian embryonic gonad. *Physiological Reviews*, 66, 71-117.
- CAHILL, L. P. & MAULEON, P. (1980a) Influences Of Season, Cycle And Breed On Follicular-Growth Rates In Sheep. *Journal Of Reproduction And Fertility*, 58, 321-328.
- CAHILL, L. P. & MAULEON, P. (1980b) Influences of season, cycle and breed on follicular growth rates in sheep. *Journal of Reproduction and Fertility*, 58, 321-328.
- CAHILL, L. P. & MAULEON, P. (1981) A study of the population of primordial and small follicles in the sheep. *Journal of Reproduction and Fertility*, 61, 201-206.
- CALDANI, M., ANTOINE, M., BATAILLER, M. & DUITTOZ, A. (1995) Ontogeny Of GnRh Systems. *Journal Of Reproduction And Fertility*, 147-162.
- CAMPBELL, B. K., BAIRD, D. T., MCNEILLY, A. S. & SCARAMUZZI, R. J. (1990a) Ovarian secretion rates and peripheral concentrations of inhibin in normal and

- androstenedione-immune ewes with an autotransplanted ovary. *J Endocrinol*, 127, 285-96.
- CAMPBELL, B. K., GORDON, B. M., TSONIS, C. G. & SCARAMUZZI, R. J. (1995a) The effect of acute immuno-neutralisation of inhibin in ewes during the early luteal phase of the oestrous cycle on ovarian hormone secretion and follicular development. *J Endocrinol*, 145, 479-90.
- CAMPBELL, B. K., MCNEILLY, A. S., PICTON, H. M. & BAIRD, D. T. (1990b) The effect of a potent gonadotrophin-releasing hormone antagonist on ovarian secretion of oestradiol, inhibin and androstenedione and the concentration of LH and FSH during the follicular phase of the sheep oestrous cycle. *J Endocrinol*, 126, 377-84.
- CAMPBELL, B. K. & SCARAMUZZI, R. J. (1995) Effect of acute immunoneutralization of inhibin in ewes during the late luteal phase of the oestrous cycle on ovarian hormone secretion and follicular development during the subsequent follicular phase. *J Reprod Fertil*, 104, 337-45.
- CAMPBELL, B. K., SCARAMUZZI, R. J., EVANS, G. & DOWNING, J. A. (1991a) Increased Ovulation Rate In Androstenedione-Immune Ewes Is Not Due To Elevated Plasma-Concentrations Of Fsh. *Journal Of Reproduction And Fertility*, 91, 655-666.
- CAMPBELL, B. K., SCARAMUZZI, R. J. & WEBB, R. (1995a) Control of antral follicle development and selection in sheep and cattle. *J Reprod Fertil Suppl*, 49, 335-50.
- CAMPBELL, B. K., SCARAMUZZI, R. J. & WEBB, R. (1995b) Control of Antral Follicle Development and Selection in Sheep and Cattle. *Journal of Reproduction and Fertility*, Suppl. 49, 335-350.
- CARATY, A. & SKINNER, D. C. (1999) Progesterone priming is essential for the full expression of the positive feedback effect of estradiol in inducing the preovulatory gonadotropin-releasing hormone surge in the ewe. *Endocrinology*, 140, 165-170.
- CARDENAS, H., MCCLURE, K. E. & POPE, W. F. (1993) Luteal Function And Blastocyst Development In Ewes Following Treatment With PGF(2)Alpha And GnRh. *Theriogenology*, 40, 865-872.
- CARDOZO, J. A., FERNANDEZ-JUAN, M., CEBRIAN-PEREZ, J. A. & MUINO-BLANCO, T. (2008) Identification of RSVPI4 and RSVPI20 Components by Two-dimensional Electrophoresis and Western-blotting. *Reproduction in Domestic Animal*, 43, 15-21.
- CARLIOS, J. H., BRUCE, K., CAMPBELL & DAVID, T. (1997) Follicular dynamics and ovarian steroid secretion in sheep during the follicular and early luteal phase of the oestrous cycle. *Biol Reprod*, 56, 483-488.
- CARLSSON, B., BERGH, C., BENTHAM, J., OLSSON, J.-H., NORMAN, M. R., BILLIG, H., ROOS, P. & HILLENSJO, T. (1992) Expression of functional growth hormone receptors in human granulosa cells. *Human Reproduction*, 7, 1205-1209.
- CARRILLO, F., HERNANDEZ-CERON, J., OROZCO, V., HERNANDEZ, J. A. & GUTIERREZ, C. G. (2007) A single dose of bovine somatotropin 5 days before the end of progestin-based estrous synchronization increases prolificacy in sheep. *Animal Reproduction Science*, 102, 31-37.
- CARSON, R. S., FINDLAY, J. K., BURGER, H. G. & TROUNSON, A. O. (1979a) Gonadotrophin receptors of the ovine ovarian follicle during follicular growth and atresia. *Biol Reprod*, 21, 75-87.
- CARSON, R. S., FINDLAY, J. K., BURGER, H. G. & TROUNSON, A. O. (1979b) Gonadotropin Receptors Of The Ovine Ovarian Follicle During Follicular-Growth And Atresia. *Biology Of Reproduction*, 21, 75-87.

- CASAS, E., FREKING, B. A. & LEYMASTER, K. A. (2005) Evaluation of Dorset, Finnsheep, Romanov, Texel, and Montadale breeds of sheep: V. Reproduction of Flewes in spring mating seasons *Journal Animal Science*, 83, 2743-2751.
- CECCONI, S., BARBONI, B., COCCIA, M. & MATTIOLI, M. (1999) In Vitro Development of Sheep Preantral Follicles. *Biology of Reproduction*, 60, 594-601.
- CELIS, J., OSTERGAARD, M., JENSEN, N., GROMOVA, I., RASMUSSEN, H. AND GROMOV, P. (1998) Human and mouse proteomic databases: novel resources in the protein. *Febs Letters*, 430, 64-72.
- CHANNING, C., P., (1970) Influence of the *in vivo* and *in vitro* hormonal environment upon luteinization of granulosa cells in tissue *Prog Horm Res*, 26, 589-622.
- CHEMINEAU, P., DAVEAU, A., LOCATELLI, A. & MAURICE, F. (1993) Ram-induced short luteal phase: effects of hysterectomy and cellular composition of the corpus luteum. *Rep. Nutri and Develop*, 33, 253-261.
- CHOI, Y. H., CARNEVALE, E. M., SEIDEL, G. E. & SQUIRES, E. L. (2001) Effects of gonadotropins on bovine oocytes matured in TCM-199. *Theriogenology*, 56, 661-670.
- CHUN, S. Y. & HSUEH, A. J. W. (1998) Paracrine mechanisms of ovarian follicle apoptosis. *Journal of Reproductive Immunology*, 39, 63-75.
- CLARK, D. E., TISDALL, D. J., FIDLER, A. E. & MCNATTY, K. P. (1996) Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries. *Journal of Reproduction and Fertility*, 106, 329-335.
- COGNIE, Y., BENOIT, F., POULIN, N., KHATIR, H. & DRIANCOURT, M. A. (1998) Effect of follicle size and of the FecB Booroola gene on oocyte function in sheep. *J Reprod Fertil*, 112, 379-86.
- CONLEY, A. & HINSHELWOOD, M. (2001) Mammalian aromatases.
- COONROD, S. A., WRIGHT, P. W. & HERR, J. C. (2002) Oolemmal proteomics. *J Reprod Immunol*, 53, 55-65.
- CORTHALS, L., WASINGER, C., HOCHSTRASSER, F. AND CHARLES J. (2000) The dynamic range of protein expression: A challenge for proteomic research. *Electrophoresis*, 21, 1104-1115.
- COX, R., MARTIN, J. T. & SHINOZUK, H. (1973) Studies on acute methionine toxicity .2. inhibition of ribonucleic-acid synthesis in guinea-pig liver by methionine and ethionine. *Laboratory Investigation*, 29, 54-59.
- CRISP, T. M. (1992) Organization of the ovarian follicle and events in its biology: oogenesis, ovulation or atresia. *Mutation Research*, 296, 89-106.
- CROSBY, T. F., BOLAND, M. P. & GORDON, I. (1991) Effects of progestagen treatments on the incidence of estrus and pregnancy rates in ewes. *Animal Reproduction Science*, 24, 109-118.
- CULLER, M. D. & NEGROVILAR, A. (1987) Pulsatile Follicle-Stimulating-Hormone Secretion Is Independent Of Luteinizing-Hormone-Releasing Hormone (Lhrh) - Pulsatile Replacement Of Lhrh Bioactivity In Lhrh-Immunoneutralized Rats. *Endocrinology*, 120, 2011-2021.
- CUMMING, I. A., BUCKMASTER, J. M., BLOCKEY, M. A., GODING, J. R., WINFIELD, C. G. & BAXTER, R. W. (1973) Constancy of interval between luteinizing hormone release and ovulation in the ewe. *Biol Reprod*, 9, 24-9.
- CUPP, A. S., STUMPF, T. T., KOJIMA, F. N., WERTH, L. A., WOLFE, M. W., ROBERSON, M. S., KITOK, R. J. & KINDER, J. E. (1995) Secretion Of Gonadotropins Change During The Luteal-Phase Of The Bovine Estrous-Cycle In The

- Absence Of Corresponding Changes In Progesterone Or 17-Beta-Estradiol. *Animal Reproduction Science*, 37, 109-119.
- D'ALESSANDRO, A. G., MARTEMUCCI, G. & TAIBI, L. (2005) How the FSH/LH ratio and dose numbers in the p-FSH administration treatment regimen, and insemination schedule affect superovulatory response in ewes. *Theriogenology*, 63, 1764-1774.
- DA SILVA, P., AITKEN, R. P., RHIND, S. M., RACEY, P. A. & WALLACE, J. M. (2001) Influence of placentally mediated fetal growth restriction on the onset of puberty in male and female lambs. *Reproduction*, 122, 375-383.
- DAVIES, C. G. (1988a) Factors affecting embryo mortality in ewe lambs *Animal Science*. Aberystwyth, Wales.
- DAVIES, J. S. & RUEDA, C. B. (2002) The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. *Frontiers in Bioscience* 7, 1949- 1978.
- DAVIES, M. C. & BECK, N. F. (1992) Plasma hormone profiles and fertility in ewe lambs given progestagen supplementation after mating. *Theriogenology*, 38, 513-26.
- DAVIES, M. C. G. (1988b) Factors affecting embryo mortality in ewe lambs. *Department of Agriculture*. Aberystwyth, Wales.
- DAVIES, M. C. G. & BECK, N. F. G. (1993) A comparison of plasma prolactin, LH and progesterone concentrations during estrus and early-pregnancy in ewe lambs and ewes. *Animal Production*, 57, 281-286.
- DAVIES, M. C. G. & BECK, N. F. G. (2003) A comparison of plasma growth hormone, insulin, free fatty acid and glucose concentrations during oestrus and early pregnancy in Clun Forest ewe lambs and ewes. *Small Ruminant Research*, 48, 127-134.
- DEL VECCHIO, R. P., SUTHERLAND, W. D. & SASSER, R. G. (1996) Bovine luteal cell production in vitro of prostaglandin E(2), oxytocin and progesterone in response to pregnancy-specific protein B and prostaglandin F-2 alpha *Journal Of Reproduction and Fertility* 107, 131-136.
- DENAMUR, R. (1974) Luteotrophic factors in the sheep. *J Reprod Fertil*, 38, 251-9.
- DICKERSON, G. E. & LASTER, D. B. (1975) Breed, heterosis and environmental influences on growth and puberty in ewe lambs. *Journal Of Animal Science*, 41, 1-9.
- DIELEMAN, S. J., HENDRIKSEN, P. J. M., VIUFF, D., THOMSEN, P. D., HYTTEL, P., KNIJN, H. M., WRENZYCKI, C., KRUIP, T. A. M., NIEMANN, H., GADELLA, B. M., BEVERS, M. M. & VOS, P. (2002) Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology*, 57, 5-20.
- DIXON, A. B., KNIGHTS, M., PATE, J. L., LEWIS, P. E. & INSKEEP, E. K. (2006) Reproductive performance of ewes after 5-day treatment with intravaginal inserts containing progesterone in combination with injection of prostaglandin F2α. *Reproduction in Domestic Animals*, 41, 142-148.
- DOWNING, J. M. (1980) Studies on attainment of puberty and reproductive performance in Clun Forest ewe lambs *University College of Wales, Aberystwyth. PhD Thesis*.
- DRIANCOURT, M. A. (2001) Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. *Theriogenology*, 55, 1211-39.
- DRIANCOURT, M. A., FAIR, T. & REYNAUD, K. (1998) Oocytary apoptosis. *Contraception Fertility Sexualite*, 26, 522-527.
- DRIANCOURT, M. A., GORMON, T., THANH, L. P. & BOOMAROV, O. (1996) Analysis by two-dimensional electrophoresis of proteins secreted by sheep ovarian follicles: Effects of the Fec(B) gene, follicle size and atresia. *Journal Of Reproduction And Fertility*, 107, 69-77.

- DRIANCOURT, M. A., REYNAUD, K. & SMITZ, J. (2001a) Differences in follicular function of 3-month-old calves and mature cows. *Reproduction*, 121, 463-474.
- DRIANCOURT, M. A., REYNAUD, K. & SMITZ, J. (2001b) Differences in follicular function of 3-month-old calves and mature cows.
- DUBY, R. T., DAMIANI, P., LOONEY, C. R., FISSORE, R. A. & ROBL, J. M. (1996) Prepubertal calves as oocyte donors: Promises and problems. *Theriogenology*, 45, 121-131.
- DUCIBELLA, T., DUFFY, P., REINDOLLAR, R. & SU, B. (1990) Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging in vivo. *Biology of Reproduction*, 43, 870-876.
- DUCKER, M. J. & BOWMAN, J. C. (1970) Photoperiodism In Ewe .3. Effects Of Various Patterns Of Increasing Daylength On Onset Of Anoestrus In Clun Forest Ewes. *Animal Production*, 12, 465-&.
- DYRMUNDSSON, O., R. (1973a) Puberty and reproductive performance in sheep. 1. Ewe lambs *Animal breeding Abstracts*, 41, 272-289.
- DYRMUNDSSON, O., R., (1973b) Puberty and early reproductive performance in sheep. 1. Ewe lambs. *Animal Breeding Abst*, 41, 237.
- DYRMUNDSSON, O., R., (1983) The influence of environmental factors on the attainment of puberty in ewe lambs. . *Sheep production*. (ed. W. Haresign), pp. 393-408. Butterworths, London.
- DYRMUNDSSON, O., R., (1981) Natural factors affecting puberty and reproductive performance in ewe lambs: a review. *Livestock Production Science*, 8, 55-65.
- EARL, D., IRVIN, B. AND ARMSTRONG, D. T. (1994) Development of techniques for the production of viable embryos from six to seven week old lambs. . *Aust Soc Anim Production*, 20, 428.
- EBLING, F. J. P. (2005) The neuroendocrine timing of puberty. *Reproduction*, 129, 675-683.
- ECKHOLM, C., HILLENSJO, I. & ISAKSSON, O. G. (1981) Gonadotropin releasing hormone agonists stimulate oocyte meiosis and ovulation in hypophysectomized rats. *Endocrinology*, 108, 2022-2024.
- EL-BATTAWY, K. A. (1998) Further studies on postpartum anestrus in buffaloes with special reference to the level of prostaglandin alpha 2. Cairo University.
- EL-BATTAWY, K. A. (2006) Reproductive and endocrine characteristics of delayed pubertal ewe-lambs after melatonin and L-tyrosine administration. *Reproduction in Domestic Animals*, 41, 1-4.
- ENGLAND, B. G., DAHMER, M. K. & WEBB, R. (1999) Relationships between follicular size and antral fluid steroid concentrations at 3 stages of the estrous cycle in the ewe *Biology of Reproduction*, 24, 1068- 1075.
- EPPIG, J. J., SCHULTZ R. M., O'BRIEN M AND CHESNEL F (1994) Relationship between developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. *Development of Biology*, 49, 1-9.
- EPPIG, J. J., SCHULTZ, R. M., O'BRIEN, M. & CHESNEL, F. (1994) Relationship between developmental programs controlling nuclear and cytoplasmic maturation oocytes. *Development Biology*, 164, 1-9.
- ESPEY, L. L. (1994) Current Status of the Hypothesis That Mammalian Ovulation is Comparable to an Inflammatory Reaction. *Biology of Reproduction*, 50, 233-238.
- ESPEY, L. L. & LIPNER, H. (1994) Ovulation. IN KNOBIL, E. & NEILL, J. D. (Eds.) *The Physiology of Reproduction*. New York, Raven Press.

- EVANS, A. C. (2003) Ovarian follicle growth and consequences for fertility in sheep. *Anim Reprod Sci*, 78, 289-306.
- EVANS, A. C., ADAMS, G. P. & RAWLINGS, N. C. (1994) Follicular and hormonal development in prepubertal heifers from 2 to 36 weeks of age. *Journal Reproduction and Fertility* 102, 463-470.
- EVANS, A. C. O., DUFFY, P., HYNES, N. & BOLAND, M. P. (2000) Waves of follicle development during the estrous cycle in sheep. *Theriogenology*, 53, 699-715.
- EVANS, A. C. O., FLYNN, J. D., DUFFY, P., KNIGHT, P. G. & BOLAND, M. P. (2002) Effects of ovarian follicle ablation on FSH, oestradiol and inhibin A concentrations and growth of other follicles in sheep. *Reproduction*, 123, 59-66.
- FABER-NYS, C., AND VERNIER, G.. (1987) Development and use of a method for quantifying female sexual behaviour in ewes. . *Appl. Anim. Behav. Sc.*, , 17, 289-304.
- FAIRCHILD, D. L. & PATE, J. L. (1991) Modulation of bovine luteal cell synthetic capacity by interferon-gamma. *Biol Reprod.*, 44, 357-63.
- FAIRCLOUGH, R., MOORE, L. & MCGOWAN, L. T. (1980) Temporal relationship between plasma-concentrations of "13,14-dihydro-15-keto-prostaglandin f and neurophysin i-ii around luteolysis in sheep *Prostaglandins*, 20, 199-208.
- FARIN, C. E., MOELLER, C. L., MAYAN, H., GAMBONI, F., SAWYER, H. R. & NISWENDER, G. D. (1988) Effect of luteinizing hormone and human chorionic gonadotropin on cell populations in the ovine corpus luteum. 38, 413-421.
- FINDLAY, J. K., RUSSELL, D. L., DOUGHTON, B., TSONIS, C. G., BORCHERS, C. & FORAGE, R. G. (1994) Effect of active immunization against the amino-terminal peptide (alpha-n) of the alpha-43-kDa subunit of inhibin (alpha-43) on fertility of ewes. *Reprod Fertil Development* 6, 265-267.
- FINK, G. (1988) Gonadotrophin secretion and its control. In: *Knobil E, Neill J, eds. The Physiology of reproduction. New York, NY: Raven Press*, 1349-1377.
- FITZGERALD, J., MICHEL, F. & BUTLER, W. R. (1982) Growth and sexual maturation in ewes: the role of photoperiod, diet and temperature on growth rate and the control of prolactin, thyroxine and luteinizing hormone secretion. *J Anim Sci*, 55, 1431-40.
- FLINT, A. P. & SHELDRIK, E. L. (1983) Evidence for a systemic role for ovarian oxytocin in luteal regression in sheep.
- FLINT, A. P. F., SHELDRIK, E. L., MCCANN, T. J. & JONES, D. S. C. (1990) Luteal oxytocin: characteristics and control of synchronous episodes of oxytocin and PGF2[alpha] secretion at luteolysis in ruminants. *Domestic Animal Endocrinology*, 7, 111.
- FLYNN, J. D., DUFFY, P., BOLAND, M. P. & EVANS, A. C. (2000) Progestagen synchronisation in the absence of a corpus luteum results in the ovulation of a persistent follicle in cyclic ewe lambs. *Anim Reprod Sci*, 62, 285-96.
- FORREST, P. A. & BICHARD, M. (1974) Analysis of production records from a lowland sheep flock .2. flock statistics and reproductive-performance *Anim Prod*, 19, 25-32.
- FORSDIKE, R. A., HARDY, K., BULL, L., STARK, J., WEBBER, L. J., STUBBS, S., ROBINSON, J. E. & FRANKS, S. (2007) Disordered follicle development in ovaries of prenatally androgenized ewes.
- FORTUNE, J. E. (1994) Ovarian Follicular Growth and Development in Mammals. *Biology of Reproduction*, 50, 225-232.
- FORTUNE, J. E. & QUIRKE, S. M. (1988) Regulation of steroidogenesis in bovine preovulatory follicles *Journal Animal Science Supplement*. 1 66, 1-8.

- FOSTER, D. L. (1981) Mechanism for delay of 1st ovulation in lambs born in the wrong season (Fall). *Biology Of Reproduction*, 25, 85-92.
- FOSTER, D. L., EBLING, F. J., CLAYPOOL, L. E., WOOD, R. I., ADEL, T. E. & SCHRAMM, W. (1989) Amplitude modulation of the nightly melatonin rise in the neonatal lamb and the subsequent timing of puberty. 40, 920-928.
- FOSTER, D. L. & KARSCH, F. J. (1975) Development of mechanism regulating preovulatory surge of luteinizing-hormone in sheep. *Endocrinology*, 97, 1205-1209.
- FOSTER, D. L., KARSCH, F. J., OLSTER, D. H., RYAN, K. D. & YELLON, S. M. (1986) Determinants of puberty in seasonal breeder. *Hormone Research*, 42, 331-384.
- FOSTER, D. L. & YELLON, S. M. (1985) Photoperiodic time measurement is maintained in undernourished lambs with delayed puberty. *J Reprod Fertil*, 75, 203-208.
- FOSTER, D. L., YELLON, S. M., EBLING, F. J. P. & CLAYPOOL, L. E. (1988) Are ambient short-day cues necessary for puberty in a short-day breeder. *Biology Of Reproduction*, 38, 821-829.
- FOSTER, D. L., YELLON, S. M. & OLSTER, D. H. (1985) Internal And External Determinants Of The Timing Of Puberty In The Female. *Journal Of Reproduction And Fertility*, 75, 327-344.
- FOSTER, J. P., LAMMING, G. E. & PETERS, A. R. (1980) Short-term relationships between plasma LH, FSH and progesterone concentrations in post-partum dairy cows and the effect of Gn-RH injection. 59, 321-327.
- FOWLER, P. A. & MCNEILLY, A. S. (1997) Maternal pituitary gonadotroph function in relation to GnRH receptor and LH beta mRNA content during pregnancy in ewes *Journal of Reproduction and Fertility* 110, 267-278.
- FOWLKES, J. L., THRAILKILL, K. M., GEORGENASCIMENTO, C., ROSENBERG, C. K. & SERRA, D. M. (1997) Heparin-binding, highly basic regions within the thyroglobulin type-1 repeat of insulin-like growth factor (IGF)-binding proteins (IGFBPs) -3, -5, and -6 inhibit IGFBP-4 degradation. *Endocrinology*, 138, 2280-2285.
- FRANCE, J. T., MASON, J. I., MAGNESS, R. R., MURRY, B. A. & ROSENFELD, C. R. (1987) Ovine placental aromatase: Studies of activity levels, kinetic characteristics and effects of aromatase inhibitors. *Journal of Steroid Biochemistry*, 28, 155-160.
- FRANCHIMONT, P., DEMOULIN, A. & VALCKE, J. C. (1988) Endocrine, Paracrine and Autocrine Control of Follicular Development. *Hormone and Metabolic Research*, 20, 193-203.
- FREEMAN, M. E., BUTCHER, R. L. & FUGO, N. W. (1970) Alteration of oocytes and follicles by Delayed Ovulation. *Biology Reproduction*, 2, 209-215.
- FREI, R. E., SCHULTZ, G. A. & CHURCH, R. B. (1989) Qualitative and quantitative changes in protein synthesis occur at the 8-16-cell stage of embryogenesis in the cow. *Journal of Reproduction and Fertility* 86, 637-641.
- FROESCH, E. R., SCHMID, C., SCHWANDER, J. & ZAPF, J. (1985) Actions of insulin-like growth factors. . *Annu. Rev. Physiol.*, 47, 443-467.
- GANDOLFI, F., MILANESI, E., POCAR, P., LUCIANO, A. M., BREVINI, T. A., ACOCELLA, F., LAURIA, A. & ARMSTRONG, D. T. (1998) Comparative analysis of calf and cow oocytes during in vitro maturation. *Mol Reprod Dev*, 49, 168-75.
- GASKINS, C. T., SNOWDER, G. D., WESTMAN, M. K. & EVANS, M. (2005) Influence of body weight, age, and weight gain on fertility and prolificacy in four breeds of ewe lambs. *J Anim Sci*, 83, 1680-9.
- GEVAERT, K. & VANDEKERCKHOVE, J. (2000) Protein identification methods in proteomics. *Electrophoresis. Review*, 21, 1145-1154.

- GHARIB, S. D., WIERMAN, M. E., SHUPNIK, M. A., . & CHIN, W. W. (1990) Molecular biology of pituitary gonadotropins. *Endocr Rev.*, 11, 177-199.
- GIBBONS, J. R., KOT, K., THOMAS, D. L., WILTBANK, M. C. & GILTHER, O. J. (1999) Follicular and fsh dynamics in ewes with a history of high and low ovulation rates. *Theriogenology*, 52, 1005-1020.
- GILBERT, S. F. (1997) *Developmental Biology*. Sinauer Publisher, Sunderland, Massachusetts.
- GINTHER, O. J. (1974) Internal regulation of physiological processes through local venoarterial pathways: a review. *J Anim Sci*, 39, 550-64.
- GINTHER, O. J., KOT, K. & WILTBANK, M. C. (1995) Associations Between Emergence Of Follicular Waves And Fluctuations In Fsh Concentrations During The Estrous-Cycle In Ewes. *Theriogenology*, 43, 689-703.
- GIRMUS, R. L. & WISE, M. E. (1991) Direct pituitary effects of estradiol and progesterone on luteinizing-hormone release, stores and subunit messenger ribonucleic-acids. *Biology Of Reproduction*, 45, 128-134.
- GIRSH, E., WANG, W., MAMLUK, R., ARDITI, F., FRIEDMAN, A., MILVAE, R. A. & MEIDAN, R. (1996) Regulation of endothelin-1 expression in the bovine corpus luteum: Elevation by prostaglandin F-2 alpha. *Endocrinology*, 137, 5191-5196.
- GLASS, J. D., FITZ, T. A. & NISWENDER, G. D. (1984) Cytosolic Receptor For Estradiol In The Corpus-Luteum Of The Ewe - Variation Throughout The Estrous-Cycle And Distribution Between Large And Small Steroidogenic Cell-Types. *Biology Of Reproduction*, 31, 967-974.
- GOFF, A. K., PONTBRIAND, D. & SIROIS, J. (1987) Oxytocin stimulating of plasma 15-keto-13,14- dihydroprostaglandin alpha during the oestrous cycle and pregnancy in the mare. . *J.Reprod. Fertil. Supplement.*, 35.
- GOMEZ, M. C., CATT, J. W., EVANS, G. & MAXWELL, W. M. C. (1998) Cleavage, development and competence of sheep embryos fertilized by intracytoplasmic sperm injection and in vitro fertilization. *Theriogenology*, 49, 1143-1154.
- GONG, J. G., BRAMLEY, T. & WEBB, R. (1991) The effect of recombinant bovine somatotropin on ovarian-function in heifers - follicular populations and peripheral hormones. *Biology Of Reproduction*, 45, 941-949.
- GONG, J. G., BRAMLEY, T. A. & WEBB, R. (1993) The effect of recombinant bovine somatotropin on ovarian follicular-growth and development in heifers. *Journal Of Reproduction And Fertility*, 97, 247-254.
- GONZALEZ-ANOVER, P., ENCINAS, T., VEIGA-LOPEZ, A., AMMOUN, I., CONTRERAS, I., ROS, J. M., ARIZNAVARRETA, C., TRESGUERRES, J. A. F. & GONZALEZ-BULNES, A. (2007) Effects of breed on follicular dynamics and oestradiol secretion during the follicular phase in sheep. *Reprod Dom Anim*, 42, 29-33.
- GONZALEZ-BULNES, A., SOUZA, C. J. H., CAMPBELL, B. K. & BAIRD, D. T. (2004) Systemic and intraovarian effects of dominant follicles on ovine follicular growth. *Animal Reproduction Science*, 84, 107-119.
- GONZALEZ DE BULNES, A., GARCIA-GARCIA, R. M., CASTELLANOS, V., SANTIAGO-MORENO, J., ARIZNAVARRETA, C. & DOMINGUEZ. (2003) Influence of maternal environment on the number of transferable embryos obtained in response to superovulatory FSH treatments in ewes. *Reprod. Nutr. Dev*, 43, 17-28.
- GOODMAN, R. L., BITTMAN, E. L., FOSTER, D. L. & KARSCH, F. J. (1981) The endocrine basis of the synergistic suppression of luteinizing hormone by estradiol and progesterone *Endocrinology*, 109, 1414-1417.

- GOODMAN, R. L. & KARSCH, F. J. (1980) Pulsatile secretion of luteinizing-hormone - differential suppression by ovarian-steroids. *Endocrinology*, 107, 1286-1290.
- GORDON, I. (1967) Aspects of reproduction and neonatal mortality in ewe lambs and adult sheep *J. Dep. Agric. Fish., Irl.*, 64, 76-127.
- GORDON, I. (1997) *Controlled reproduction in sheep and goats.*, CAB, Slough.
- GORDON, I. (2003) Laboratory production of cattle Embryos, second ed. *In: Biotechnology in Agriculture Series, No 27 CAB International.*, 27.
- GORDON, I. (2004) Pathophysiology of renal function and its effect on isotope studies in the workup of hydronephrosis. *World Journal of Urology*, 22, 411- 416.
- GORDON, I. & . (1996) *Controlled Reproduction in Farm Animals Series. Reproduction in sheep and goats*, CAB International, Wallingford.
- GOSDEN, R. G. & TELFER, E. (1987) Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships. *Journal of Zoology*, 211, 169-175.
- GRAVES, P. E., PIERCE, K. L., BAILEY, T. J., RUEDA, B. R., GIL, D. W., WOODWARD, D. F., YOOL, A. J., HOYER, P. B. & REGAN, J. W. (1995) Cloning of a receptor for prostaglandin F2 alpha from the ovine corpus luteum. *Endocrinology*, 136, 3430-6.
- GRAZUL-BILSKA, A. T., REDMER, D. A., JABLONKA-SHARIFF, A., BIONDINI, M. E. & REYNOLDS, L. P. (1995) Proliferation and progesterone production of ovine luteal cells from several stages of the estrous cycle: effects of fibroblast growth factors and luteinizing hormone. *Can J Physiol Pharmacol*, 73, 491-500.
- GRAZUL-BILSKA, A. T., REDMER, D. A. & REYNOLDS, L. P. (1997b) Cellular interactions within the corpus luteum. *Seminars in Reproductive Endocrinology*, 15, 383-393.
- GRAZUL-BILSKA, A. T., REYNOLDS, L. P., BILSKI, J. J. & REDMER, D. A. (2001a) Effects of second messengers on gap junctional intercellular communication of ovine luteal cells throughout the estrous cycle.
- GRAZUL-BILSKA, A. T., REYNOLDS, L. P., BILSKI, J. J. & REDMER, D. A. (2001b) Effects of Second Messengers on Gap Junctional Intercellular Communication of Ovine Luteal Cells Throughout the Estrous Cycle. *Biol Reprod*, 65, 777-783.
- GRAZUL-BILSKA, A. T., REYNOLDS, L. P. & REDMER, D. A. (1997) Gap junctions in the ovaries. *Biol Reprod.*, 57, 947-957.
- GRAZULBILSKA, A. T., REDMER, D. A. & REYNOLDS, L. P. (1996) Effects of luteinizing hormone and prostaglandin F-2 alpha on gap junctional intercellular communication of ovine luteal cells throughout the estrous cycle. *Endocrinology*, 5, 225-233.
- GRAZYNA PTAK, P. L., MARIA DARIA DATTENA, MARIAN TISCHNER, AND PIETRO CAPPAL (1999) Offspring from One-Month-Old lambs: Studies on the development capability of prepubertal Oocytes. *Biol Reprod*, 61, 1568-1574.
- GREALY, M., DISKIN, M. G. & SREENAN, J. M. (1996) Protein content of cattle oocytes and embryos from the two-cell to the elongated blastocyst stage at day 16. *Journal Of Reproduction And Fertility*, 107, 229-233.
- GREENWALD, G. S. & ROY, S. K. (1994) Follicular development and its control. . IN ED. KNOBIL, E. A. N., J. D. (Ed.) *physiology of reproduction*. Raven Press, Ltd., New York.
- GULYAS, B. J. (1975) Re-examination of the cleavage patterns in eutherian mammalian eggs: rotation of the blastomere pairs during second cleavage in the rabbit. *Journal. Experimental. Zoology*, 193, 235-248.
- HAFEZ, E. S. E. (1952) Studies on the breeding season and reproduction of ewe. *Journal Agriculture Science* 42, 189.

- HAFEZ, E. S. E. (1993) Folliculogenesis, Egg Maturation, and Ovulation. IN HAFEZ, E. S. E. (Ed.) *Reproduction in Farm Animals*. Sixth ed. Philadelphia, Lea & Febiger.
- HAMPL, A. & EPPIG, J. J. (1995) Analysis of the mechanism(s) of metaphase-i arrest in maturing mouse oocytes. *Development* 121, 925-933.
- HAMRA, A. H., MCNALLY, J. W., MARCEK, J. M., CARLSON, K. M. & WHEATON, J. E. (1989) Comparison Of progesterone sponges, cronolone sponges and controlled internal-drug release dispensers on fertility in anestrous ewes. *Animal Reproduction Science*, 18, 219-226.
- HAMRA, A. M. & BRYANT, M. J. (1979) Reproductive-performance during mating and early-pregnancy in young female sheep. *Animal Production*, 28, 235-243.
- HARE, L. & BRYANT, M. J. (1985) Ovulation rate and embryo survival in young ewes mated either at puberty or at the 2nd or 3rd estrus. *Animal Reproduction Science*, 8, 41-52.
- HARESIGN, W. (1983) *Sheep reproduction*, London.
- HARESIGN, W. (1992) Manipulation of reproduction in sheep. *J.Reprod. Fertil Suppl*, 45, 127-139.
- HASTIE, P. M. & HARESIGN, W. (2006a) Expression of mRNAs encoding insulin-like growth factor (IGF) ligands, IGF receptors and IGF binding proteins during follicular growth and atresia in the ovine ovary throughout the oestrous cycle. *Anim Reprod Sci*, 92, 284-99.
- HASTIE, P. M. & HARESIGN, W. (2006b) A role for LH in the regulation of expression of mRNAs encoding components of the insulin-like growth factor (IGF) system in the ovine corpus luteum. *Anim Reprod Sci*, 96, 196-209.
- HASTIE, P. M., ONAGBESAN, O. M. & HARESIGN, W. (2004) Co-expression of messenger ribonucleic acids encoding IGF-I, IGF-II, type I and II IGF receptors and IGF-binding proteins (IGFBP-1 to-6) during follicular development in the ovary of seasonally anoestrous ewes. *Animal Reproduction Science*, 84, 93-105.
- HAUGER, R. L., KARSCH, F. J. & FOSTER, D. L. (1977) New Concept For Control Of Estrous-Cycle Of Ewe Based On Temporal Relationships Between Luteinizing-Hormone, Estradiol And Progesterone In Peripheral Serum And Evidence That Progesterone Inhibits Tonic Lh-Secretion. *Endocrinology*, 101, 807-817.
- HAY, M. F. & MOOR, R. M. (1973) Graafian follicle of sheep - relationships between gonadotropins, steroid production, morphology and oocyte maturation. *Annales De Biologie. Animale. Biochimie. Biophysique, Supplement-1*, 13, 241.
- HEAP, R. B., FLEET, I. R. & HAMON, M. (1985) Prostaglandin F-2 α is transferred from the uterus to the ovary in the sheep by lymphatic and blood. *Reproduction and Fertility*, 645-656.
- HELMER, S. D. & BRITT, J. H. (1987) Hormone-secretion and characteristics of estrous cycles after treatment of heifers with human chorionic-gonadotropin or prostaglandin-F2-alpha during corpus-luteum formation. *Journal Of Animal Science*, 64, 782-789.
- HENDERSON, K. M., MCNEILLY, A. S. & SWANSTON, I. A. (1982) Gonadotrophin and steroid concentrations in bovine follicular fluid and their relationship to follicle size. *Journal of Reproduction and Fertility*, 65, 467-473.
- HERBISON, A. E. (1998) Multimodal influence of estrogen upon gonadotrophin-releasing hormone neurons *Endocrinology Review*, 19, 302-330.
- HIRSHFIELD, A. N. (1991) Development of Follicles in the Mammalian Ovary. *International Review of Cytology*, 124, 43-101.
- HIRSHFIELD, A. N. (1991a) Development of Follicles in the Mammalian Ovary. *International Review of Cytology*, 124, 43-101.

- HIRSHFIELD, A. N. & MIDGLEY, A. R. (1978) Morphometric analysis of follicular development in the rat. *Biology of Reproduction*, 19, 597-605.
- HIXON, J. E. & FLINT, A. P. (1987) Effects of a luteolytic dose of oestradiol benzoate on uterine oxytocin receptor concentrations, phosphoinositide turnover and prostaglandin F-2 alpha secretion in sheep. *Journal of Reproduction and fertility* 79, 457-467.
- HORTON, E. W. & AND POYSER, N. L. (1976) Uterine luteolytic hormone: A physiological role for prostaglandin alfa2 *Physiological Reviews*, 56, 595- 651.
- HSU, S. Y. & HSUEH, A. J. W. (1997) Hormonal regulation of apoptosis - An ovarian perspective. *Trends in Endocrinology and Metabolism*, 8, 207-213.
- HSUEH, A. J. W., ADASHI, E. Y., JONES, P. B. C. & WELSH, T. H. (1984) Hormonal regulation of the differentiation of cultured ovarian granulosa-clles. *Endocr Rev.*, 5, 76-127.
- HSUEH, A. J. W., BILLIG, H. & TSAFRIRI, A. (1994) Ovarian Follicle Atresia - a Hormonally Controlled Apoptotic Process. *Endocrine Reviews*, 15, 707-724.
- HU, J., BRAILEANU, G. T. & MIRANDO, M. A. (2001) Oxytocin stimulates prostaglandin alpha2 secretion from porcine endometrial cells through activation of calcium-dependent protein kinase C. *Prostaglandins*, 65, 85-101.
- HUET, C., MONGET, P., PISSELET, C. & MONNIAUX, D. (1997) Changes in extracellular matrix components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep. *Biology of Reproduction*, 56, 1025-1034.
- HUGGINS, J. P., PELTON, J. T. & MILER, R. C. (1993) The structure and specificity of endothelin receptors: their importance in physiology and medicine. *Pharmacology and Therapeutics.*, 59, 55-123.
- HULET, C. V., WIGGINS, E. L. AND ERCANBRACK, S. K. (1969) Estrus in range ewe lambs and its relationship to lifetime reproductive performance. *Journal Of Animal Science*, 28, 246-252.
- HUNTER, M. G., SOUTHEE, J. A. & LAMMING, G. E. (1988) Function of abnormal corpora-lutea invitro after gnrh-induced ovulation in the anestrous ewe. *Journal Of Reproduction And Fertility*, 84, 139-148.
- HUNTER, M. G., SOUTHEE, J. A., MCLEOD, B. J. & HARESIGN, W. (1986) Progesterone pretreatment has a direct effect on gnrh induced preovulatory follicles to determine their ability to develop into normal corpora-lutea in anestrous ewes. *Journal Of Reproduction And Fertility*, 76, 349-363.
- HUNTER, R. H. F. (1980) In: *Physiology and Technology of Reproduction in Female Domestic Animals*. Academic Press, London.
- HURWITZ, A. & ADASHI, E. Y. (1992) Ovarian Follicular Atresia as an Apoptotic Process - a Paradigm for Programmed Cell Death in Endocrine Tissues. *Molecular and Cellular Endocrinology*, 84, C19-C23.
- HURWITZ, A., RUUTIAINENALTMAN, K., MARZELLA, L., BOTERO, L., DUSHNIK, M. & ADASHI, E. Y. (1996) Follicular atresia as an apoptotic process: Atresia-associated increase in the ovarian expression of the putative apoptotic marker sulfated glycoprotein-2. *Journal of the Society for Gynecologic Investigation*, 3, 199-208.
- HWA, V., O H, Y. & ROSENFELD, R. G. (1999) The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocrine Reviews.*, 20, 761-787.
- I'ANSON, H., TERRY, S. K., LEHMAN, M. N. & FOSTER, D. L. (1997) Regional differences in the distribution of gonadotropin-releasing hormone cells between rapidly growing and growth-restricted prepubertal female sheep. *Endocrinology*, 138, 230-236.

- IRVINE, G. E., CASAGRANDE, V. A. & NORTON, T. T. (1993) Center/surround relationships of magnocellular parvocellular and koniocellular relay cells in primate lateral geniculate nucleus. *Visual Neuroscience*, 10, 363-373.
- JABLONKA-SHARIFF, A., GRAZUL-BILSKA, A. T., REDMER, D. A. & REYNOLDS, L. P. (1993) Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle. *Endocrinology*, 133, 1871-1879.
- JAINUDEEN, M. R., WAHID, H. & HAFEZ, E. S. E. (2000) Sheep and goats. IN HAFEZ, E. S. E. & HAFEZ, B. (Eds.) *Reproduction in farm animals*. 7th ed., Lippincott Williams and Wilkins.
- JAMESON, J. L. & HOLLENBERG, A. N. (1993) Regulation of chorionic gonadotropin gene expression. *Endocr Rev.*, 14, 203-221.
- JI, I., SLAUGHTER, R. G., ELLIS, J. A., JI, T. H. & MURDOCH, W. J. (1991) Analyses of ovine corpora lutea for tumor necrosis factor mRNA and bioactivity during prostaglandin-induced luteolysis. *Mol Cell Endocrinol*, 81, 77-80.
- JOHNSON, S. K., DAILEY, R. A., INSKEEP, E. K. & LEWIS, P. E. (1996) Effect of peripheral concentrations of progesterone on follicular growth and fertility in ewes. *Domestic Animal Endocrinology*, 13, 69-79.
- JOHANSSON, I. D., HART, I. C. & BUTLERHOGG, B. W. (1985) The effects of exogenous bovine growth-hormone and bromocriptine on growth, body development, fleece weight and plasma-concentrations of growth-hormone, insulin and Prolactin in female lambs. *Animal Production*, 41, 207-217.
- JOLLY, P. D., SMITH, P. R., HEATH, D. A., HUDSON, N. L., LUN, S., STILL, L. A., WATTS, C. H. & MCNATTY, K. P. (1997a) Morphological evidence of apoptosis and the prevalence of apoptotic versus mitotic cells in the membrana granulosa of ovarian follicles during spontaneous and induced atresia in ewes. *Biology Of Reproduction*, 56, 837-846.
- JOLLY, P. D., TISDALL, D. J., DEATH, G., HEATH, D. A., LUN, S., HUDSON, N. L. & MCNATTY, K. P. (1997b) Granulosa cell apoptosis, aromatase activity, cyclic adenosine 3',5'-monophosphate response to gonadotropins, and follicular fluid steroid levels during spontaneous and induced follicular atresia in ewes. *Biology of Reproduction*, 56, 830-836.
- JORGENSEN, J. C., KANNISTO, P., LIEBERG, F., OTTESEN, B., OWMAN, C. & SCHMIDT, G. (1991) The influence of neuropeptide Y and norepinephrine on ovulation in the rat ovary. *Peptides*, 12, 975-982.
- JOUBERT, D. M. (1963) Puberty in female farm animals. *Animal Breeding Abstract*.
- JOYCE, I. M., KHALID, M. & HARESIGN, W. (1998) Growth hormone priming as an adjunct treatment in superovulatory protocols in the ewe alters follicle development but has no effect on ovulation rate. *Theriogenology*, 50, 873-884.
- JUENGEL, J. L., WILTBANK, M. C., MEBERG, B. M. & NISWENDER, G. D. (1996) Regulation of steady-state concentrations of messenger ribonucleic acid encoding prostaglandin F2 alpha receptor in ovine corpus luteum. *Biol Reprod*, 54, 1096-102.
- JUNEJA, S. C., BARR, K. J., ENDERS, G. C. & KIDDER, G. M. (1999) Defects in the germ line and gonads of mice lacking connexin43. *Biology Of Reproduction*, 60, 1263-1270.
- KAIPIA, A. & HSUEH, A. J. W. (1997) Regulation of ovarian follicle atresia. *Annual Review of Physiology*, 59, 349-363.
- KAISER, G. G., SINOWATZ, F. & PALMA, G. A. (2001) Effects of growth hormone on female reproductive organs. *Jornal of Veterinary Medicine Series*, 30, 265-71.

- KARSCH, F. J., BITTMAN, E. L., FOSTER, D. L., GOODMAN, R. L., LEGAN, S. J. & ROBINSON, J. E. (1984) Neuroendocrine basis of seasonal reproduction. *Recent Progress Hormone Research*, 40, 185-232.
- KARSCH, F. J., CUMMINS, J. T., THOMAS, G. B. & CLARKE, I. J. (1987) Steroid Feedback Inhibition Of Pulsatile Secretion Of Gonadotropin-Releasing-Hormone In The Ewe. *Biology Of Reproduction*, 36, 1207-1218.
- KARSCH, F. J. & EVANS, N. P. (1996) Feedback actions of estradiol on GnRH secretion during the follicular phase of the oestrous cycle. *Acta Neurobiologiae Experimentalis* 56, 715-725.
- KARSCH, F. J., GOODMAN, R. L. & LEGAN, S. J. (1980) Feedback Basis Of Seasonal Breeding - Test Of An Hypothesis. *Journal Of Reproduction And Fertility*, 58, 521-535.
- KATSKA, L. & RYNSKA, B. (1998) The isolation and in vitro culture of bovine preantral and early antral follicles of different size classes. *Theriogenology*, 50, 213-222.
- KAULFUSS, K. H., BRABANT, S. & BLUME, K. (1994) Possibilities and limits of the ultrasound ovary diagnosis in sheep. *Bildgebung/Imaging Suppl.*, 61., 98.
- KEENAWAY, D. J., DUNSTAN, E. A. & STAPLES, L. D. (1987) Photoperiodic control of the onset of breeding activity and fecundity in ewes. *Journal Reproduction and Fertility Supplement* 34, 187-199.
- KENNAWAY, D. J., GILMORE, T. A. & SEAMARK, R. F. (1982) Effect of melatonin feeding on serum prolactin and gonadotropin-levels and the onset of seasonal estrous cyclicity in sheep. *Endocrinology*, 110, 1766-1772.
- KENNEDY, J. P., WORTHING, C. A. & COLE, E. R. (1974) Postnatal-development of ovary and uterus of merino lamb. *Journal of Reproduction and Fertility*, 36, 275-&.
- KESKINTEPE, L. & BRACKETT, B. G. (1996) In vitro developmental competence of in vitro-matured bovine oocytes fertilized and cultured in completely defined media. *Biology Reproduction*, 55, 333-339.
- KHALID, M. & HARESIGN, W. (1996) Relationships between concentrations of ovarian steroids, insulin-like growth factor-1 and IGF-binding proteins during follicular development in the ewe. *Animal Reproduction Science*, 41, 119-129.
- KHALID, M., HARESIGN, W. & LUCK, M. R. (2000) Secretion of IGF-1 by ovine granulosa cells: effects of growth hormone and follicle stimulating hormone. *Anim Reprod Sci*, 58, 261-72.
- KHAN, T. H. (2000) The effects of gonadotrophin supplementation on embryo mortality in ewes and ewe lambs. *Institute of Rural Studies*. . Aberystwyth, Wales.
- KHAN, T. H., BECK, N. F. G. & KHALID, M. (1999) The effects of hCG treatment on day 12 post mating on conceptus growth and fertility in ewes and ewe lambs. *J.Reprod. Fertil.*, 34, Abstr No. 23.
- KHAN, T. H., BECK, N. F. G. & KHALID, M. (2007) The effects of GnRH analogue (buserelin) or hCG (Chorulon) on Day 12 of pregnancy on ovarian function, plasma hormone concentrations, conceptus growth and placentation in ewes and ewe lambs. *Animal Reproduction Science*, 102, 247-257.
- KHAN, T. H., BECK, N. F. G., MANN, G. E. & KHALID, M. (2006) Effect of post-mating GnRH analogue (buserelin) treatment on PGF2[alpha] release in ewes and ewe lambs. *Animal Reproduction Science*, 95, 107-115.
- KINDAHL, H., ALFONSO, R., CORT, N. & EINARSSON, S. (1982.) Release of prostaglandin alfa2 during parturition in the sow. . *Vet Med.*, 29., 504- 510.

- KINDER, J. E., BERGFELD, E. G., WEHRMAN, M. E., PETERS, K. E. & KOJIMA, F. N. (1995) Endocrine basis for puberty in heifers and ewes. *J Reprod Fertil Suppl*, 49, 393-407.
- KNICKERBOCKER, J. J., WILTBANK, M. C. & NISWENDER, G. D. (1988) Mechanisms of luteolysis in domestic livestock. *Domestic Animal Endocrinology*, 5, 91-107.
- KNIJN, H. M., GJORRET, J. O., VOS, P., HENDRIKSEN, P. J. M., VAN DER WEIJDEN, B. C., MADDOX-HYTTEL, P. & DIELEMAN, S. J. (2003) Consequences of in vivo development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos. *Biology Of Reproduction*, 69, 1371-1378.
- KOCHHAR, H. P., WU, B., MORRIS, L. H., BUCKRELL, B. C., POLLARD, J. W., BASRUR, P. K. & KING, W. A. (2002a) Maturation status, protein synthesis and developmental competence of oocytes derived from lambs and ewes. *Reprod Domest Anim*, 37, 19-25.
- KOCHHAR, H. P. S., WU, B., MORRIS, L. H. A., BUCKRELL, B. C., POLLARD, J. W., BASRUR, P. K. & KING, W. A. (2002b) Maturation Status, Protein Synthesis and Developmental Competence of Oocytes Derived from Lambs and Ewes. 37, 19-25.
- KOHNO.H., OKAMOTO.C., IIDA. K., TAKEDA.T., ETSUSHI. K, KAWASHIMA. C., MIYAMOTO. A. & FUKUI.Y. (2005) Comparison of estrus induction and subsequent fertility with two different intravaginal devices in ewes during the non-breeding season. *Journal of Reproduction and Development*, 51, 805-812.
- KRARUP, T., PEDERSEN, T. & FABER, M. (1969) Regulation of oocyte growth in the mouse ovary. *Nature*, 224, 187-188.
- KRUIP, T. A. M. & DIELEMAN, S. J. (1985) Steroid hormone concentrations in the fluid of bovine follicles relative to size, quality and stage of the oestrus cycle. *Theriogenology*, 24, 395-408.
- KRUIP, T. A. M. & DIELEMAN, S. J. (1989) Intrinsic and extrinsic factors influencing steroid production in vitro by bovine follicles. *Theriogenology*, 31, 531-544.
- KRUIP, T. A. M., DIELEMAN, S. J. & MOOR, R. M. (1979) Steroid-production by bovine follicles invitro - influence of size, stage of cycle and culture system. *Annales De Biologie Animale Biochimie Biophysique*, 19, 1537-1545.
- KRZYMOWSKI, T. & STEFANCZYK-KRZYMOWSKA, S. (2008) The role of the endometrium in endocrine regulation of the animal oestrous cycle.
- LAFRANCE, M. & GOFF, A. K. (1985) Effect of pregnancy on oxytocin induced release of prostaglandin alpha2 in heifers. *Biol Reprod.*, 33, 1113-1119.
- LAHLOUKASSI, A. & MARIANA, J. C. (1984) Ovarian follicular-growth during the estrous-cycle in 2 breeds of ewes of different ovulation rate, the dman and the timahdite. *Journal Of Reproduction And Fertility*, 72, 301-310.
- LAND, R. B. (1970) Number of oocytes present at birth in ovaries of pure and finnish landrace cross blackface and welsh sheep. *Journal Of Reproduction And Fertility*, 21, 517-&.
- LAND, R. B. (1978) Reproduction in young sheep - some genetic and environmental sources of variation. *Journal Of Reproduction And Fertility*, 52, 427-436.
- LANE, H. W., STRENGTH, R., JOHNSON, J. & WHITE, M. (1991) Effect of chemical form of selenium on tissue glutathione peroxidase activity in developing rats.
- LASTER, D. B., DICKERSO, G. E. & GLIMP, H. A. (1972) Factors affecting reproduction in ewe lambs *Journal of Animal Science* 35, 79-&.
- LASTER., D. B. D., G. E. AND GLIMP., H. A. (1972.) FACTORS AFFECTING REPRODUCTION IN EWE LAMBS. *JOURNAL OF ANIMAL SCIENCE.*, 35., 79-&.

- LEDDA, S., BOGLIOLO, L., CALVIA, P., LEONI, G. & NAITANA, S. (1997) Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. *J Reprod Fertil*, 109, 73-8.
- LEDDA, S., BOGLIOLO, L., LEONI, G. & NAITANA, S. (1999a) Production and lambing rate of blastocysts derived from in vitro matured oocytes after gonadotropin treatment of prepubertal ewes. *J Anim Sci*, 77, 2234-9.
- LEDDA, S., BOGLIOLO, L., LEONI, G. & NAITANA, S. (1999b-a) Follicular size affects the meiotic competence of in vitro matured prepubertal and adult oocytes in sheep. *Reprod Nutr Dev*, 39, 503-8.
- LEDDA, S., BOGLIOLO, L., LEONI, G. & NAITANA, S. (1999b-b) Follicular size affects the meiotic competence of in vitro matured prepubertal and adult oocytes in sheep. *Reproduction Nutrition Development*, 39, 503-508.
- LEDDA, S., BOGLIOLO, L., CALVIA, P., LEONI, G. AND NAITANA, S. (1996) Meiotic progression and development competence of oocytes collected from juvenile and adult ewes. *Reproduction and fertility*, 109, 73-78.
- LEFTSSON, E. & DYRMUNDSSON, O., R. (1990) Duration of oestrus in Icelandic ewes and ewe lambs *Burvisindi*, 4, 71-76.
- LEGAN, S. J. & KARSCH, F. J. (1980) Photoperiodic control of seasonal breeding in ewes - modulation of the negative feedback action of estradiol. *Biology Of Reproduction*, 23, 1061-1068.
- LEIBO, S. P. & LOSKUTOFF, N. M. (1993) Cryobiology of invitro-derived bovine embryos. *Theriogenology*, 39, 81-94.
- LEVESQUE, J., AND SIRARD (1994) Protein in oocytes from calves and adult cows before maturation: relationship with their development capacity. *Reprod Nutr Dev*, 34, 133-139.
- LEYMARIE, P. & MARTAL., J. (1993) The corpus luteum from cycle to gestation. IN THIBAUT, C., LEVASSEUR., M., AND HUNTER, (Ed.) *Reproduction in Mammals and Management*.
- LIEBERMANN, J., SCHAMS, D. & MIYAMOTO, A. (1996) Effects of local growth factors on the secretory function of bovine corpus luteum in vitro. *Rep. Fertil. Dev.*, 8, 1003-1011.
- LOHUIS, M. M. (1995) Potential benefits of bovine embryo-manipulation technologies to genetic-improvement programs. *Theriogenology*, 43, 51-60.
- LONERGAN, P., DINNYES, A., FAIR, T., YANG, X, AND BOLAND, M. (2000) bovine oocyte and embryo development following meiotic inhibition with butyrolactone I. . *Mol Reprod Dev*, 57, 204-209.
- LONGRIGG, W. (1961) Sheep management. Prog. Rep. Exp. Husb. Fms. *Exp Hort Stas*, 8-10
- LOUIS, T. M., PARRY, D. M., ROBERSON, J. S., THORBURN, G. D. & GHALLIS, J. R. G. (1977) Effects of exogenous progesterone and oestradiol on prostaglandin F and 13, 14-dihydro-15oxoprostaglandin alpha2 concentrations in uteri and plasma of ovariectomized ewes. *Journal of Endocrinology*, 73, 427-439.
- LUCY, M. C., CURRAN, T. L., COLLIER, R. J. & COLE, W. J. (1994) Extended function of the corpus luteum and earlier development of the second follicular wave in. *Theriogenology*, 41, 561-672.
- LUSSIER, J. G., MATTON, P. & DUFOUR, J. J. (1987) Growth rates of follicles in the ovary of the cow. *J Reprod Fertil*, 81, 301-307.

- MACIEL, G. A. R., BARACAT, E. C., BENDA, J. A., MARKHAM, S. M., HENSINGER, K., CHANG, R. J. & ERICKSON, G. F. (2004) Stockpiling of Transitional and Classic Primary Follicles in Ovaries of Women with Polycystic Ovary Syndrome. *Journal of Clinical Endocrinology and metabolism*, 89, 5321-5327.
- MACLAUGHLIN, S. M., WALKER, S. K., KLEEMANN, D. O., SIBBONS, J. P., TOSH, D. N., GENTILI, S., COULTER, C. L. & MCMILLEN, I. C. (2007) Impact of periconceptional undernutrition on adrenal growth and adrenal insulin-like growth factor and steroidogenic enzyme expression in the sheep fetus during early pregnancy. *Endocrinology*, 148, 1911-20.
- MALVEN, P. V. & HANSEL, W. (1964) Ovarian function in dairy heifers following hysterectomy. *Journal . Dairy Science.* , 47, 1388-1393.
- MANN, G. E., MCNEILLY, A. S. & BAIRD, D. T. (1992) Hormone production in vivo and in vitro from follicles at different stages of the oestrous cycle in the sheep. *J Endocrinol*, 132, 225-34.
- MARIANA GROKE MARQUES, A. C. N., VIVIANE PURRI DE OLIVEIRA, ANIBAL BALLAROTTI NASCIMENTO, HELOISA VASCONCELOS AMARAL CAETANO, CAMILLA MOTA MENDES, MARCO ROBERTO BOURG MALLO, MARCELLA PECORA MILAZZOTTO, MAYRA ELENA ORTIZ D' AVILA ASSUMPCAO, JOSE ANTONIO VISINTIN (2007) In vitro maturation of pig oocytes with different media, hormone and meiosis inhibitors *Anim Reprod Sci*, 97, 375-381.
- MARION, G. B., GIER, H. T. & CHOUDARY, J. B. (1968) Micromorphology of the bovine ovarian follicular system. *Journal of Animal Science*, 27, 451-465.
- MARUO, T., HAYASHI, M., MATSU, O., UEDA, Y. & MORIKAWA, H. (1988) Comparison of the facilitative roles of insulin and insulin-like growth factor I in the functional. . *Scandinavian University Press.*, 117, 230-240.
- MCCRACKEN, J. A., BAIRD, D. T. & GODING, J. R. (1971) Factors affecting the secretion of steroids from the transplanted ovary in the sheep. *Recent Prog Horm Res.*, 27, 537-582.
- MCCRACKEN, J. A., CARLSON, J. C., GLEW, M. E., GODING, J. R., BAIRD, D. T., GREEN, K. & SAMUELSSON, B. (1972) Prostaglandin alfa2 identified as a luteolytic hormone in sheep. *Nature.*, 238, 129-134.
- MCCRACKEN, J. A., CUSTER, E. E., ELDERING, J. A. & ROBINSON, A. G. (1996) The central oxytocin pulse generator: a pacemaker for the ovarian cycle. *Acta Neurobiologiae Experimentalis (Wars)*, 56, 819-32.
- MCCRACKEN, J. A., GLEW, M. E. & SCARAMUZZI, R. J. (1970) Corpus luteum regression induced by prostaglandin in F2-alpha. *J Clin Endocrinol Metab*, 30, 544-6.
- MCDONALD, L. E. (1980) *Veterinary endocrinology and reproduction*, Bailliere Tindall, London.
- MCGUIRE, W. J., JUENGEL, J. L. & NISWENDER, G. D. (1994a) Protein-Kinase-C 2nd Messenger System Mediates The Antisteroidogenic Effects Of Prostaglandin-F2-Alpha In The Ovine Corpus-Luteum In-Vivo. *Biology Of Reproduction*, 51, 800-806.
- MCGUIRE, W. J., JUENGEL, J. L. & NISWENDER, G. D. (1994b) Protein kinase C second messenger system mediates the antisteroidogenic effects of prostaglandin F2 alpha in the ovine corpus luteum in vivo. *Biol Reprod*, 51, 800-6.
- MCKENZIE, F. F. & TERRILL, C. E. (1937) Estrus, ovulation and related phenomena in the ewe. *Mo. Agr Exp. Sta. Res. Bull.*, 264, 1-88.

- MCLAREN, A. (2000) Germ and somatic cell lineages in the developing gonad. *Molecular and Cellular Endocrinology*, 163, 3-9.
- MCMILLAN, W. H. & MCDONALD, M. F. (1985) Survival of fertilized ova from ewe lambs and adult ewes in the uteri of ewe lambs. *Animal Reproduction Science*, 8, 235-240.
- MCNATTY, K. P., HEATH, D. A., HUDSON, N. L., BALL, K. & CONDELL, L. (1992a) Concentrations of immunoreactive inhibin in ovarian and peripheral venous plasma and follicular fluid of Booroola ewes that are homozygous carriers or non-carriers of the FecB gene. *J Reprod Fertil*, 95, 489-502.
- MCNATTY, K. P., HUDSON, N., GIBB, M., BALL, K., HENDERSON, K. M., HEATH, D. A., LUN, S. & KIEBOOM, L. E. (1985) FSH influences follicle viability, oestradiol biosynthesis and ovulation rate in Romney ewes. *Journal of Reproduction and Fertility* 75, 121-131.
- MCNATTY, K. P., SMITH, P., HUDSON, N. L., HEATH, D. A., LUN, S. & O, W. S. (1992b) Follicular development and steroidogenesis. IN SJOBERG, N., HAMBERGER, L., JANSEN, P. O., OWMAN, C. & COELINGH, H. I. J. (Eds.) *Local Regulation of Ovarian Function*. Carnforth, Parthenon Publishing Group.
- MCNEILLY, A. S., CROW, W. J. & FRASER, H. M. (1992) Suppression of pulsatile luteinizing hormone secretion by gonadotrophin-releasing hormone antagonist does not affect episodic progesterone secretion or corpus luteum function in ewes. *J Reprod Fertil*, 96, 865-74.
- MCNEILLY, A. S., HUNTER, M., LAND, R. B. & FRASER, H. M. (1981) Inadequate corpus-luteum function after the Induction of ovulation in anestrus ewes by LH-RH or and Lh-Rh agonist. *Journal of Reproduction and Fertility*, 63, 137-144.
- MCNEILLY, A. S., OCONNELL, M. & BAIRD, D. T. (1982) INDUCTION OF OVULATION AND NORMAL LUTEAL FUNCTION BY PULSED INJECTIONS OF LUTEINIZING-HORMONE IN ANESTRUS EWES. . *ENDOCRINOLOGY*, 110, 1292-1299.
- MILLAR, R. P., ZL, L. U., PAWSON, A. J., FLANAGAN, C. A., MORGAN, K. & MAUDSLEY, S. R. (2004) Gonadotrophin-releasing hormone receptors. *Endocr. Rev.*, 25, 235-725.
- MILLER, E. A., GE, Z., HEDGPETH, V. & GADSBY, J. E. (2003) Steroidogenic responses of pig corpora lutea to insulin-like growth factor I (IGF-I) throughout the oestrous cycle. *Reproduction*, 125, 241-249.
- MILVAE, R. A., HINCKLEY, S. T. & CARLSON, J. C. (1996) Luteotropic and luteolytic mechanisms in the bovine corpus luteum *Theriogenology*, 45, 1327-1349.
- MIWA, M., TOKUDA, H., TSUSHITA, K., KOTOYORI, J., TAKAHASHI, Y., OZAKI, N., KOZAWA, O. & OISO, Y. (1990) Involvement of pertussis toxin-sensitive GTP-binding protein in prostaglandin alpha2 induced phosphoinositide hydrolysis in osteoblast-like cells. *Biochem. Biophys. Res. Commun.*, 171, 1229-1235.
- MOENTER, S. M., CARATY, A., LOCATELLI, A. & KARSCH, F. J. (1991) Pattern of gonadotropin-releasing-hormone (Gnrh) secretion leading up to ovulation in the ewe - existence of a preovulatory Gnrh surge. *Endocrinology*, 129, 1175-1182.
- MONGET, P., MONNIAUX, D., PISSELET, C. & DURAND, P. (1993) Changes In Insulin-Like Growth Factor-I (Igf-I), Igf-II, And Their Binding-Proteins During Growth And Atresia Of Ovine Ovarian Follicles. *Endocrinology*, 132, 1438-1446.
- MONNIAUX, D. (1987) Short-term effects of Fsh invitro on granulosa-cells of individual sheep follicles. *Journal Of Reproduction And Fertility*, 79, 505-515.

- MONNIAUX, D., CHUPIN, D. & SAUMANDE, J. (1983) Superovulatory responses of cattle. *Theriogenology*, 19, 55-81.
- MONNIAUX, D., MONGET, P., BESNARD, N., HUET, C. & PISSELET, C. (1997) Growth factors and antral follicular development in domestic ruminants. *Theriogenology*, 47, 3-12.
- MONNIAUX, D. & PISSELET, C. (1992) Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-I and follicle-stimulating hormone in vitro. *Biol Reprod*, 46, 109-19.
- MOOR, R. M. & CROSBY, I. M. (1986) Protein requirements for germinal vesicle breakdown in ovine oocytes. *Journal Of Embryology Experiment Morphology*, 94, 207-220.
- MOOR, R. M. & CROSBY, I. M. (1987) Cellular origin, hormonal regulation and biochemical characteristics of polypeptides secreted by Graafian follicles of sheep. *Journal Reproduction . Fertility* 79, 469-483.
- MOOR, R. M., DAI, Y., LEE, C. & FULKA, J., JR. (1998) Oocyte maturation and embryonic failure. *Human Reproduction Update*, 4, 223-226.
- MOOR, R. M., DAI, Y., LEE C AND FULKA J. (1998) Oocyte maturation and embryonic failure. *Human Reproduction Update*, 4, 223-246.
- MOOR, R. M., HAY, M. F., DOTT, H. M. & CRAN, D. G. (1978) Macroscopic identification and steroidogenic function of atretic follicles in sheep. *Journal Endocrinology*, 77, 309-318.
- MOOR, R. M., MARY, F., HAY, J., MCINTOSH, E. A. & CALDWELL, B. V. (1973) Effect of gonadotrophins on the production of steroids by sheep ovarian follicles cultured in vitro *Journal Endocrinology*, 58, 599-611.
- MOOR, R. M. & TROUNSON, A. O. (1977) Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity.
- MOOR, R. M. & TROUNSON, A. O. (1997) Hormonal and follicular factors affecting of sheep oocytes in vitro and their subsequent development capacity. *Reprod fertil* 49.
- MORENO, J. S., BRUNET, A. G., BULNES, A. G., VILLAR, D. & SEBASTIAN, A. L. (2000) Attainment of Puberty in the European Mouflon (*Ovis gmelini musimon*) and the Domestic Manchega Ewe (*Ovis aries*). *Reproduction in Domestic Animal*, 35, 49-52.
- MOTILK J, A. K. M. (1990) Cell cycle aspects of growth and maturation of mammalian oocytes. *Mol Reprod Dev*, 27, 366-375.
- MOUDGAL, N. R., SHETTY, G., SELVARAJ, N. & BHATANAGAR, A. S. (1996) Use of specific aromatase inhibitor for determining whether there is a role for oestrogen in follicle/oocyte maturation, ovulation and preimplantation embryo development *Journal Reproduction and Fertility Suppl*, 50, 69-81.
- MURDOCH, W. J., DAILEY, R. A. & INSKEEP, E. K. (1981) Preovulatory Changes in Prostaglandin-E2 and Prostaglandin-F2- Alpha in Ovine Follicles. *Journal of Animal Science*, 53, 192-205.
- MURDOCH, W. J. & DUNN, T. G. (1983) Luteal function after ovulation blockade by intra-follicular injection of indomethacin in the ewe. *Journal of Reproduction and Fertility*, 69, 671-675.
- NAGATANI, S., GUTHIKONDA, P., THOMPSON, R. C., TSUKAMURA, H., MAEDA, K. I. & FOSTER, D. L. (1998) Evidence for GnRH regulation by leptin: Leptin administration prevents reduced pulsatile LH secretion during fasting. *Neuroendocrinology*, 67, 370-376.
- NANDI, S., GIRISH KUMAR, V., MANJUNATHA, B. M., RAMESH, H. S. & GUPTA, P. S. P. (2008) Follicular fluid concentrations of glucose, lactate and pyruvate in buffalo and

- sheep, and their effects on cultured oocytes, granulosa and cumulus cells. *Theriogenology*, 69, 186-196.
- NEILL, J. D. (2002) Miniereview. GnRH and GnRH receptor genes in the human genome. *Endocrinology.*, 143., 737-743.
- NEILL, J. D. (2002.) Miniereview. GnRH and GnRH receptor genes in the human genome. *Endocrinology.*, 143., 737-743.
- NEPHEW, K. P., CARDENAS, H., MCCLURE, K. E., OTT, T. L., BAZER, F. W. & POPE, W. F. (1994) Effects of administration of human chorionic gonadotropin or progesterone before maternal recognition of pregnancy on blastocyst development and pregnancy in sheep. *Journal of Animal Science*, 72, 453-458.
- NEPHEW, K. P., MCCLURE, K. E., OTT, T. L., DUBOIS, D. H., BAZER, F. W. & POPE, W. F. (1991) Relationship Between Variation In Conceptus Development And Differences In Estrous-Cycle Duration In Ewes. *Biology Of Reproduction*, 44, 536-539.
- NICOLA, N. A. (1994) *Guid to Cytokines and Their Receptors*, Oxford University Press, Oxford.
- NISHIZUKA, Y. (1986) The molecular homogeneity of protein kinase C and its implications for cellular regulation. *Nature*, 334, 661-665.
- NISWENDER, G. D., JUENGEL, J. L., MCGUIRE, W. J., BELFIORE, C. J. & WILTBANK, M. C. (1994) Luteal function: the estrous cycle and early pregnancy. *Biol Reprod.*, 50, 239-247.
- NISWENDER, G. D., JUENGEL, J. L. & SILVA, P. J. (2000) Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews*, 80, 1-29.
- NISWENDER, G. D., SCHWALL, R. H., FITZ, T. A., FARIN, C. E. & SAWYER, H. R. (1985) Regulation of luteal function in domestic ruminants: new concepts. *Recent Prog Horm Res*, 41, 101-51.
- NOTHNICK, W. B. & PATE, J. L. (1990) Interleukin-1-beta is a potent stimulator of prostaglandin synthesis in bovine luteal cells. *Biology Reproduction*, 43, 898-903.
- NOWAK, R. & RODWAY, R. G. (1985) Effect of intravaginal implants of melatonin on the onset of ovarian activity in adult and prepubertal ewes. *Journal of Reproduction and fertility*, 74, 287-293.
- NUOVO, G. J. (1996) The foundations of successful RT in situ PCR. *Front Biosci*, 1, c4-15.
- O'BRIEN, J. K., CATT, S. L., IRELAND, K. A., MAXWELL, W. M. C. & EVANS, G. (1997) *In vitro* and *in vivo* developmental capacity of oocytes from prepubertal and adult sheep. *Theriogenology*, 47, 1433-1443.
- O'BRIEN, J. K., DWARTE, D., RYAN, J. P., MAXWELL, W. M. & EVANS, G. (1996) Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. *Reprod Fertil Dev*, 8, 1029-37.
- O'CALLAGHAN, D. & BOLAND, M. (1999) Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants *Animal Science*, 68, 299-314.
- OHTANI, M., KOBAYSHI, S., MIYAMOTO, A., HAYASHI, K. & FUKUI, Y. (1998) Real-time relationships between intraluteal and plasma concentrations of endothelin, oxytocin, and progesterone during prostaglandin alpha2 induced luteolysis in the cow. *Biol Reprod*, 58, 103-108.
- OKANO, A., OKUDA, K., TAKAHASHI, M. & SCHAMS, D. (1996) Oxytocin receptors in the porcine endometrium during the estrous cycle and early pregnancy. *Animal Reproduction Science*, 41, 61-70.

- OLOFSSON, J., NORJAVAARA E. & SELSTAM, G. (1992) Synthesis of prostaglandin F₂ alpha, E₂ and prostacyclin in isolated corpora lutea of adult pseudopregnant rats throughout the luteal life-span. *Prostaglandins*, 46, 151-161.
- OPPENHEIMER, S. B. (1984) Fertilization, Implantation and early Development. IN OPPENHEIMER, S. B. (Ed.) *In: Introduction to embryonic Development*. Allyn and Bacon, New York.
- ORSI, N. M., GOPICHANDRAN, N., LEESE, H. J., PICTON, H. M. & HARRIS, S. E. (2005) Fluctuations in bovine ovarian follicular fluid composition throughout the oestrous cycle. *Reproduction* 129, 219-228.
- OSMAN, P. (1985) Rate and course of atresia during follicular development in the adult cyclic rat. *Journal of Reproduction and Fertility*, 73, 261-270.
- OTOI, T., FUJII, M., TANAKA, M., OOKA, A. & SUZUKI, T. (2000) Canine oocyte diameter in relation to meiotic competence and sperm penetration. *Theriogenology*, 54, 535-542.
- OYOYO, U. A., SHAH, U. S. & MURRAY, S. A. (1997) The role of alpha1 (connexin-43) gap junction expression in adrenal cortical cell function. *Endocrinology*, 138, 5385-5396.
- PAAVOLA, L. G. & CHRISTENSEN, A. L. (1981) Characterization of granule types in luteal cells of sheep at the time of maximum progesterone secretion *Biology Reproduction*, 25, 203-215.
- PADMANABHAN, V., MCFADDEN, K., MAUGER, D. T., KARSCH, F. J. & MIDGLEY, R. (1997) Neuroendocrine control of follicle-stimulating hormone (FSH) secretion .1. Direct evidence for separate episodic and basal components of FSH secretion. *Endocrinology*, 138, 424-432.
- PALMA, G. A., CLEMENT-SENGEVALD, A. AND KREFFT, H (1993) In vitro production of cattle embryos from calf oocytes. *Theriogenology*, 39, 278.
- PAPACHRISTOFOROU, C., KOUMAS, A. & PHOTIOU, C. (2000) Seasonal effects on puberty and reproductive characteristics of female Chios sheep and Damascus goats born in autumn or in February. *Small Ruminant Research*, 38, 9-15.
- PATE, J. L. & CONDON, W. A. (1989) Regulation of steroidogenesis and cholesterol-synthesis by prostaglandin-f₂-alpha and lipoproteins in bovine luteal cells. *Reproduction and Fertility*, 87, 439-446.
- PATON, A. C. & COLLINS, W. P. (1992) Differentiation processes of granulosa cells. IN MILLIGAN, S. R. (Ed.) *Oxford Reviews of Reproductive Biology*. Oxford, Oxford University Press.
- PELLETIER, J., BODIN, L., HANOCQ, E., MALPAUX, B., TEYSSIER, J., THIMONIER, J. & CHEMINEAU, P. (2000) Association between expression of reproductive seasonality and alleles of the gene for Mel(1a) receptor in the ewe. *Biology Of Reproduction*, 62, 1096-1101.
- PEPPER, M. S., FERRARA, N., ORCI, L. & MONTESANO, R. (1991) Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochemical. Biophysical. Research. Communication*, 181, 902-906.
- PERKS, C. M., DENNINGKENDALL, P. A., GILMOUR, R. S. & WATHES, D. C. (1995) Localization of messenger ribonucleic-acids for insulin-like growth-factor-I (Igf-I), Igf-II, and the type-1 Igf receptor in the ovine ovary throughout the estrous-cycle. *Endocrinology*, 136, 5266-5273.

- PERKS, C. M., PETERS, A. R. & WATHES, D. C. (1999) Follicular and luteal expression of insulin-like growth factors I and II and the type 1 IGF receptor in the bovine ovary. *Journal Of Reproduction And Fertility*, 116, 157-165.
- PETERS, A. R., DREW, S. B., MANN, G. E., LAMMING, G. E. & BECK, N. F. (1992) Experimental and practical approaches to the establishment and maintenance of pregnancy. *J Physiol Pharmacol*, 43, 143-52.
- PETERS, H. (1978) Folliculogenesis in mammals. IN JONES, R. (Ed.) *The Vertebrate Ovary*. New York, Plenum.
- PHARRISS, B. B., CORNETTE, J. C. & GUTKNECHT, G. D. (1970) Vascular control of luteal steroidogenesis. *J Reprod Fertil Suppl.*, 10, 97-103.
- PIERCE, J. G. & PEARSON, R. F. (1981) Glycoprotein hormones: structure and function *Annual Review of Biochemistry*, 50, 465-495.
- PIQUETTE, G. N., LAPOLT, P. S., OIKAWA, M. & HSUEH, A. J. W. (1991) Regulation of Luteinizing Hormone Receptor Messenger Ribonucleic Acid Levels by Gonadotropins, Growth Factors, and Gonadotropin-Releasing Hormone in Cultured Rat Granulosa Cells. *Endocrinology*, 128, 2449-2456.
- PRATHER, R. S., SIMS, M. M. & MAUL, G. G. (1989) Nuclear lamin antigens are developmentally regulated during porcine and bovine embryogenesis *Biology of Reproduction*, 41, 123-132.
- PRESICCE, G. A., JIANG, S., SIMKIN, M., ZHANG, L., LOONEY, C. R., GODKE, R. A. & YANG, X. Z. (1997) Age and hormonal dependence of acquisition of oocyte competence for embryogenesis in prepubertal calves. *Biology Of Reproduction*, 56, 386-392.
- PTAK, G., LOI, P., DATTENA, M., TISCHNER, M. & CAPPAL, P. (1999) Offspring from one-month-old lambs: studies on the developmental capability of prepubertal oocytes. *Biology of Reproduction*, 61, 1568-1574.
- PTAK, G., TISCHNER, M., BERNABO, N. & LOI, P. (2003) Donor-dependent developmental competence of oocytes from lambs subjected to repeated hormonal stimulation. *Biol Reprod*, 69, 278-85.
- QUIRKE, J. F. (1977a) Studies related to the reproductive performance of adult and immature sheep. Dublin, National University of Ireland.
- QUIRKE, J. F. (1979b) Control of reproduction in adult ewes and ewe lambs, and estimation of reproductive wastage in ewe lambs following treatment with progestagen impregnated sponges and PMSG *Livestock Production Science*, 6, 295-305.
- QUIRKE, J. F. (1981a) Regulation of puberty and reproduction in female lambs: a review. *Livestock Production Science*, 8, 37-53.
- QUIRKE, J. F., ADAM, T. E. & HANRAHAN, J. P. (1983a) Artificial induction of puberty in ewe lambs. IN HARESIGN, W. (Ed.) *Sheep production* London, Butterworths.
- QUIRKE, J. F., ADAMS, T. E. & HANRAHAN, J. P. (1983) Artificial induction of puberty in ewe lambs. *In Sheep Production (ed. W. Haresign)*. pp. 409-429, Butterworths, London.
- QUIRKE, J. F. & GOSLING, J. P. (1978) Pre-Pubertal Plasma Luteinizing-Hormone Concentrations And Progesterone Concentrations During The Estrous-Cycle And Early-Pregnancy In Galway And Fingalway Female Lambs. *Animal Production*, 28, 1-12.
- QUIRKE, J. F., GOSLING, J. P. (1981b) Reproductive performance and plasma progesterone concentration in ewe lambs and mature ewes following treatment for synchronisation of oestrus during the breeding season. *Irish Journal of Agricultural Research*, 20, 9-20.

- QUIRKE, J. F. & HANRAHAN, J. P. (1977) Comparison of survival In uteri of adult ewes of cleaved ova from adult ewes and ewe lambs. *Journal of Reproduction and Fertility*, 51, 487-489.
- QUIRKE, J. F. & HANRAHAN, J. P. (1983) Comparison of the survival of fertilized eggs from adult ewes in the uteri of adult ewes and ewe lambs. *J Reprod Fertil*, 68, 289-94.
- QUIRKE, J. F., HANRAHAN, J. P. & GOSLING, J. P. (1979) Plasma Progesterone Levels Throughout The Estrous-Cycle And Release Of Lh At Estrus In Sheep With Different Ovulation Rates. *Journal Of Reproduction And Fertility*, 55, 37-44.
- QUIRKE, J. F., HANRAHAN, J. P. & GOSLING, J. P. (1981) Duration of oestrus, ovulation rate, time of ovulation and plasma LH, total oestrogen and progesterone in Galway adult ewes and ewe lambs. *Journal of Reproduction and fertility*, 61, 265-272.
- QUIRKE, J. F., STABENFELDT, G. H. & BRADFORD, G. E. (1985) Onset of Puberty and Duration of the Breeding Season in Suffolk, Rambouillet, Finnish Landrace, Dorset and Finn-Dorset Ewe Lambs. *J. Anim Sci.*, 60, 1463-1471.
- RAE, M. T., PALASSIO, S., KYLE, C. E., BROOKS, A. N., LEA, R. G., MILLER, D. W. & RHIND, S. M. (2001) Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction*, 122, 915-922.
- RAJKUMAR, R. R., ARGO, C. M. & RODWAY, R. G. (1992) Effect of melatonin on pulsatile release of luteinizing hormone in female lambs. *Hormone and Metabolic Research*, 24, 229-32.
- RAVINDRA, I. P., RAWLINGS, N. C., EVANS, A. C. O. & ADAMS, G. P. (1994a) Ultrasonographic Study Of Ovarian Follicular Dynamics In Ewes During The Estrous-Cycle. *Journal Of Reproduction And Fertility*, 101, 501-509.
- RAVINDRA, J. P., RAWLINGS, N. C., EVANS, A. C. & ADAMS, G. P. (1994b) Ultrasonographic study of ovarian follicular dynamics in ewes during the oestrous cycle. *J Reprod Fertil*, 101, 501-9.
- RAW, R. E. & SILVIA, W. J. (1991) Activity of phospholipase C and release of prostaglandin F2 alpha by endometrial tissue from ovariectomized ewes receiving progesterone and estradiol. *Biology of Reproduction*, 44, 404-412.
- RAWLINGS, N. C. & CHURCHILL, I. J. (1990) effect of naloxone on gonadotrophin secretion at various stages of development in the ewe lambs. *J. Reprod. Fertil.*, 89, 503-509.
- REDMER, D. A. & REYNOLDS, L. P. (1996) Angiogenesis in the ovary. *Rev. Reprod.*, 1, 182- 192.
- REICH, R., KOHEN, F., NAOR, Z. & TSAFRIRI, A. (1983) Possible involvement of lipoxigenase products of arachidonic acid pathway in ovulation. . *Prostaglandins.*, 26, 1011-20.
- REVEL, F., MERMILLOD, P., PEYNOT, N., RENARD, J, AND HEYMAN, Y. (1995) Low development capacity of in vitro matured and fertilised oocytes from calves compared with that of cows. *J. Reprod. Fertil.*, 103, 115-120.
- REYNA, J., THOMSON, P. C., EVANS, G. & MAXWELL, W. M. C. (2007) Synchrony of ovulation and follicular dynamics in merino ewes treated with GnRH in the breeding and non-breeding seasons. *Reproduction in Domestic Animals* 42, 410-417.
- REYNOLDS, L. P., KILLILEA, S. D., GRAZUL-BILSKA, A. T. & REDMER, D. A. (1994) Mitogenic factors of corpora lutea. *progress in growth factor Research.*, 5, 159-175.

- RICHARD, F. J. & SIRARD, M. A. (1996) Effects of follicular cells on oocyte maturation .2. Theca cell inhibition of bovine oocyte maturation in vitro. *Biology Of Reproduction*, 54, 22-28.
- RICHARDS, J. S. (1980) Maturation of ovarian follicles - actions and interactions of pituitary and ovarian hormones on follicular cell-differentiation. *Physiological Reviews*, 60, 51-89.
- RICHARDS, J. S. (1994) Hormonal control of gene expression in the ovary. *Endocr Rev*, 15, 725-751.
- RICHARDS, J. S. & HEDIN, L. (1988) Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. . *Annu Rev Physiol*, 50, 441-463.
- RICHARDS, J. S., RUSSELL, D. L., OCHSNER, S. & ESPEY, L. L. (2002) Ovulation: New dimensions and new regulators of the inflammatory-like response. *Annual Review of Physiology*, 64, 69-92.
- RIVERA, G. M., CHANDRASEKHER, Y. A., EVANS, A. C. O., GIUDICE, L. C. & FORTUNE, J. E. (2001) A potential role for insulin-like growth factor binding protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle. *Biology Of Reproduction*, 65, 102-111.
- RIVERA, G. M. & FORTUNE, J. E. (2003) Selection of the dominant follicle and insulin-like growth factor (IGF)-binding proteins: Evidence that pregnancy-associated plasma protein A contributes to proteolysis of IGF-binding protein 5 in bovine follicular fluid. *Endocrinology*, 144, 437-446.
- RIZOS, D., WARD, F., DUFFY, P., BOLAND, M. P. & LONERGAN, P. (2002) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev*, 61, 234-48.
- ROBINSON, J. J. (1996) Nutrition and reproduction. *Animal Reproduction Science*, 42, 25-34.
- ROBINSON, J. J., ASHWORTH, C. J., ROOKE, J. A., MITCHELL, L. M. & MCEVOY, T. G. (2006) Nutrition and fertility in ruminant livestock. *Animal Feed Science and Technology*, 126, 259-276.
- ROBINSON, J. J., WALLACE, J. M., AITKEN, R. P. & MCNEILLY, A. S. (1993) Effect of chronic treatment with a GnRH agonist to suppress pulsatile LH secretion on the ability of exogenous melatonin to advance oestrous cyclicity in ewes. *Journal Reproduction and Fertility*, 99, 601-608.
- ROBINSON, T. J. & SMITH, J. F. (1967) The time of ovulation after withdrawal of SC-9880-impregnated intervaginal sponges from cyclic merino ewes. IN ROBINSON, T. J. (Ed.) *Control of the ovarian cycle in the sheep*. Sydney., Sydney University. .
- ROCHE, J. F. (1996) Control and regulation of folliculogenesis--a symposium in perspective. *Rev Reprod*, 1, 19-27.
- ROGERS, J. T., BRIDGES, K. R., DURMOWICZ, G. P., GLASS, J., AURON, P. E. & MUNRO, H. N. (1990) Translational control during the acute phase response. Ferritin synthesis in response to interleukin-1. *J. Biol. Chem.*, 265, 14572-14578.
- ROWSON, L. E., TERVIT, H. R. & BRAND, A. (1972.) The use of prostaglandins for synchronization of oestrus in cattle. *Journal Reproduction and Ferti (Abst)*, 29, 145.
- RUBIANES, E., UNGERFELD, R., VINOLES, C., RIVERO, A. & ADAMS, G. P. (1997) Ovarian response to gonadotropin treatment initiated relative to wave emergence in ultrasonographically monitored ewes. *Theriogenology*, 47, 1479-1488.
- SAHA, S., SHIMIZU, M., GESHI, M. & IZAIKE, Y. (2000) In vitro culture of bovine preantral follicles. *Animal Reproduction Science*, 63, 27-39.

- SAUERWEIN, H., MIYAMOTO, A., GUNTHER, J., MEYER, H. H. D. & SCHAMS, D. (1992) Binding and action of insulin-like growth-factors and insulin in bovine luteal tissue during the estrous-cycle. *Journal Of Reproduction And Fertility*, 96, 103-115.
- SCARAMUZZI, R. & LAND, R. B. (1978) Estradiol levels in sheep plasma during estrous-cycle. *Journal of Reproduction and Fertility*, 53, 167-171.
- SCARAMUZZI, R. J., TURNBALL, K. E. & NANCARROW, C. D. (1980) Growth of Graafian follicles in cows following luteolysis induced by the prostaglandin F2 α analogue, cloprostenol. *Australian Journal of Biological Science*, 33, 63-69.
- SCHAMS, D. (1987) Luteal peptides and intracellular communication. *J.Reprod. Fertil. Supplement*, 34, 87-99.
- SCHENKEN, R. S., WILLIAMS, R. F. & HODGEN, G. D. (1984) Ovulation induction using "pure" follicle-stimulating hormone in monkeys. *Fertility and Sterility*, 41, 629-634.
- SCHMITT, E. J., BARROS, C. M., FIELDS, P. A., FIELDS, M. J., DIAZ, T., KLUGE, J. M. & THATCHER, W. W. (1996a) A cellular and endocrine characterization of the original and induced corpus luteum after administration of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin on day five of the estrous cycle. *Journal of Animal Science*, 74, 1915-1929.
- SCHMITT, E. J., DIAZ, T., BARROS, C. M., DE LA SOTA, R. L., DROST, M., FREDRIKSSON, E. W., STAPLES, C. R., THORNER, R. & THATCHER, W. W. (1996) Differential response of the luteal phase and fertility in cattle following ovulation of the first-wave follicle with human chorionic gonadotropin or an agonist of gonadotropin-releasing hormone. *J Anim Sci*, 74, 1074-83.
- SHELDRIK, E. L. & FLINT, A. P. (1985) Endocrine control of uterine oxytocin receptors in the ewe. *Journal of Endocrinology*, 106, 249-258.
- SHIRAZI, A. & SADEGHI, N. (2007a) The effect of ovine oocyte diameter on nuclear maturation. *Small Ruminant Research*, 69, 103-107.
- SILVIA, W. J., LEWIS, G. S., MCCracken, J. A., THATCHER, W. W. & WILSON, L. (1991) Hormonal-regulation of uterine secretion of prostaglandin-F2-Alpha during luteolysis in ruminants. *Biology Of Reproduction*, 45, 655-663.
- SIMONETTI, L., FORCADA, F., RIVERA, O. E., CAROU, N., ALBERIO, R. H., ABECIA, J. A. & PALACIN, I. (2008) Simplified superovulatory treatments in Corriedale ewes. *Animal Reproduction Science*, 104, 227-237.
- SINGH, B., BARBE, G. J. AND ARMSTRONG. (1993) Factors influencing resumption of meiotic maturation and cumulus expansion of porcine oocytes-cumulus cell complexes in vitro. *Mol Reprod Dev*, 36, 113-119.
- SIRARD, M.-A., RICHARD, F., BLONDIN, P. & ROBERT, C. (2006) Contribution of the oocyte to embryo quality. *Theriogenology*, 65, 126-136.
- SIRARD, M. A., FLORMAN, H. M., LEIBFRIED-RUTLEDGE, M. L., BARNES, F. L., SIMS, M. L. & FIRST, N. L. (1989) Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biology of Reproduction*, 40, 1257-1263.
- SKINNER, D. C. & ROBINSON, J. E. (1995) Melatonin-binding sites in the gonadotroph-enriched zona tuberalis of ewes. *J Reprod Fertil*, 104, 243-250.
- SLAVIK, T., FULKA, J. & GOLL, I. (1992) Pregnancy rate after the transfer of sheep embryos originated from randomly chosen oocytes matured and fertilized invitro. *Theriogenology*, 38, 749-756.
- SLIWOWSKA, J. H., BILLINGS, H. J., GOODMAN, R. L., COOLEN, L. M. & LEHMAN, M. N. (2004) The premammillary hypothalamic area of the ewe: anatomical

- characterization of a melatonin target area mediating seasonal reproduction. *Biology of Reproduction*, 70, 1768-1775.
- SLOTIN, C. A., HEAP, R. B., CHRISTIANSEN, J. M. & LINZELL, J. (1970) Synthesis of progesterone by the mammary gland of the goat. *Nature.*, 225, 385-386.
- SMEATON, T. C. & ROBERTSON, H. A. (1971) Studies on growth and atresia of graafian follicles in ovary of sheep. *Journal of Reproduction And Fertility*, 25, 243-&.
- SMITH, P., O, W. S., CORRIGAN, K. A., SMITH, T., LUNDY, T., DAVIS, G. H. & MCNATTY, K. P. (1997) Ovarian morphology and endocrine characteristics of female sheep fetuses that are heterozygous or homozygous for the Inverdale prolificacy gene (*fecXI*). *Biology of Reproduction*, 57, 1183-1192.
- SMITH, P., O, W. S., HUDSON, N. L., SHAW, L., HEATH, D. A., CONDELL, L., PHILLIPS, D. J. & MCNATTY, K. P. (1993a) Effects of the booroola gene (*Fecb*) on body-weight, ovarian development and hormone concentrations during fetal life. *Journal of Reproduction And Fertility*, 98, 41-54.
- SMITH, P., O, W. S., HUDSON, N. L., SHAW, L., HEATH, D. A., CONDELL, L., PHILLIPS, D. J. & MCNATTY, K. P. (1993b) Effects of the Booroola Gene (*Fec^B*) on Body Weight, Ovarian Development and Hormone Concentrations During Fetal Life. *Journal of Reproduction and Fertility*, 98, 41-54.
- SOMCHIT, A., CAMPBELL, B. K., KHALID, M., KENDALL, N. R. & SCARAMUZZI, R. J. (2007) The effect of short-term nutritional supplementation of ewes with lupin grain (*Lupinus luteus*), during the luteal phase of the estrous cycle on the number of ovarian follicles and the concentrations of hormones and glucose in plasma and follicular fluid. *Theriogenology*, 68, 1037-1046.
- SOUZA, C. J. H., CAMPBELL, B. K. & BAIRD, D. T. (1996) Follicular dynamics and ovarian steroid secretion in sheep during anoestrus. *Journal of Reproduction And Fertility*, 108, 101-106.
- STRAUSS, J. F. & PENNING, T. M. (1999) Synthesis of the sex steroid hormones: molecular and structural biology with applications to clinical practice. IN FAUSER, B. C. J. M., RUTHERFORD, A. J., STRAUSS, J. F. & VAN STEIRTEGHEM, A. (Eds.) *Molecular Biology in Reproductive Medicine*. Carnforth, Parthenon Publishing.
- SUNDERLAND, S. J., O'CALLAGHAN, D., BOLAND, M. P. & ROCHE, J. F. (1995) Effect of photoperiod before and after birth on puberty in ewe lambs. *Biology of Reproduction*, 53, 1178-1182.
- SUZUKI, K. & TAMAOKI, B. I. (1980) Postovulatory decrease in estrogen production is caused by the diminished supply of aromatizable. *Endocrinology.*, 107, 2115-2116.
- TAMBE, S. S. & NANDEDKAR, T. D. (1993) Steroidogenesis in sheep ovarian antral follicles in culture: time course study and supplementation with a precursor. *Steroids*, 58, 379-83.
- TASENDE, C., MEIKLE, A., RODRIGUEZ-PINON, M., FORSBERG, M. & GAROFALO, E. G. (2002) Estrogen and progesterone receptor content in the pituitary gland and uterus of progesterone-primed and gonadotropin releasing hormone-treated anestrus ewes. *Theriogenology*, 57, 1719-1731.
- TAYMOR, M. L. (1996) The regulation of follicle growth: some clinical implications in reproductive endocrinology. *Fertility and Sterility*, 65, 235-247.
- TESTART, J., THEBAULT, A. & SOUDERES, E. (1982) Premature ovulation after ovarian ultrasonography. *Premature ovulation after ovulation* 89, 694-700.

- THANKY, N. R., SLATER, R. & HERBISON, A. E. (2003) Sex differences in estrogen-dependent transcription of gonadotropin-releasing hormone (GnRH) gene revealed in GnRH transgenic mice. *Endocrinology*, 144, 3351-3358.
- THEODOSIADOU, E., GOULAS, P., KOUSKOURA, T. & SMOKOVITIS, A. (2004) Oestrogen and progesterone concentrations in plasma and oviductal tissue of ewes exhibiting a natural or induced oestrus. *Animal Reproduction Science*, 80, 59-67.
- THEODOSIS, D. T., WOODING, F. B., SHELDRIK, E. L. & FLINT, A. P. (1986) Ultrastructural localisation of oxytocin and neurophysin in the ovine corpus luteum. *Cell Tissue Res*, 243, 129-35.
- THIBAUT, C., SZOLLOSI, D. & GERARD, M. (1987) Mammalian oocyte maturation *Rep. Nutri and Develop*, 27, 885-896.
- THOMPSON, J. G. (2000) In vitro culture and embryo metabolism of cattle and sheep embryos - a decade of achievement. *Animal Reproduction Science*, 60, 263-275.
- TILLY, J. L. (1996) Apoptosis and ovarian function. *Reviews of Reproduction*, 1, 162-172.
- TILLY, J. L., KOWALSKI, K. I., SCHOMBERG, D. W. & HSUEH, A. J. (1992b) Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome P450 aromatase. *Endocrinology*, 131, 1670-1676.
- TILLY, J. L., TILLY, K. I. & PEREZ, G. I. (1997) The genes of cell death and cellular susceptibility to apoptosis in the ovary: A hypothesis. *Cell Death and Differentiation*, 4, 180-187.
- TORTONESE, D. J. & INSKEEP, E. K. (1992) Effects of melatonin treatment on the attainment of puberty in heifers. *Journal of Animal Science*, 70, 2822-2827.
- TSAI, S. J. & WILTBANK, M. C. (1997) Prostaglandin F-2 alpha induces expression of prostaglandin G/H synthase-2 in the ovine corpus luteum: A potential positive feedback loop during luteolysis. *Biology of Reproduction*, 57, 1016-1022.
- TSAI, S. J. & WILTBANK, M. C. (1998) Prostaglandin F-2 alpha regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. *Biology of Reproduction*, 58, 346-352.
- TSONIS, C. G., CAHILL, L. P., CARSON, R. S. & FINDLAY, J. K. (1984) Identification at the onset of luteolysis of follicles capable of ovulation in the ewe. *Journal of Reproduction And Fertility*, 70, 609-614.
- TUREK, F. W. & CAMPBELL, C. S. (1979) Photoperiodic Regulation Of Neuroendocrine-Gonadal Activity. *Biology of Reproduction*, 20, 32-50.
- TURNBULL, K. E., BRADEN, A. W. & MATTNER, P. E. (1977) The pattern of follicular growth and atresia in the ovine ovary. *Aust J Biol Sci*, 30, 229-41.
- VALASI, I., LEONTIDES, L., MENEGATOS, I. & AMIRIDIS, G. S. (2007) Oestradiol concentration as a predictor of ovarian response in FSH stimulated ewe-lambs. *Animal Reproduction Science*, 102, 145-151.
- VALASI, I., MENEGATOS, I., PAPANIKOLAOU, T., GOULAS, P. & AMIRIDIS, G. S. (2006) Oocyte pick-up in juvenile lambs affects neither onset of puberty nor their future fertility. *Theriogenology*, 66, 2144-2151.
- VAN DE LEEMPUT, E. E., VOS, P. L., ZEINSTRA, E. C., BEVERS, M. M., VAN DER WEIJDEN, G. C. & DIELEMAN, S. J. (1999) Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled preovulatory LH surge. *Theriogenology*, 52, 335-349.
- VAN DEN HURK, R., BEVERS, M. M. & BECKERS, J. F. (1997) In-vivo and in-vitro development of preantral follicles. *Theriogenology*, 47, 73-82.

- VAN DEN HURK, R., DIJKSTRA, G., VAN MIL, F. N., HULSHOF, S. C. J. & VAN DEN INGH, T. S. G. A. M. (1995) Distribution of the intermediate filament proteins vimentin, keratin, and desmin in the bovine ovary. *Molecular Reproduction and Development*, 41, 459-467.
- VANDENHURK, R., BEVERS, M. M. & BECKERS, J. F. (1997) In-vivo and in-vitro development of preantral follicles. *Theriogenology*, 47, 73-82.
- VANDENHURK, R., DIJKSTRA, G., VANMIL, F. N., HULSHOF, S. C. J. & VANDENINGH, T. (1995) Distribution of the intermediate filament proteins vimentin, keratin and desmin in the bovine ovary. *Molecular Reproduction And Development*, 41, 459-467.
- VILLAVICENCIO, A., INIGUEZ, G., JOHNSON, M. C., GABLER, F., PALOMINO, A. & VEGA, M. (2002) Regulation of steroid synthesis and apoptosis by insulin-like growth factor I and insulin-like growth factor binding protein 3 in human corpus luteum during the midluteal phase. *Reproduction*, 124, 501-508.
- VINCENT, D. L. & INSKEEP, E. K. (1986) Role of progesterone in regulating uteroovarian venous concentrations of PGF2 alpha. *Prostaglandins*, 31, 715-733.
- VINOLES, C., FORSBERG, M., BANCHERO, G. & RUBIANES, E. (2001) Effect of long-term and short-term progestagen treatment on follicular development and pregnancy rate in cyclic ewes. *Theriogenology*, 55, 993-1004.
- WALKER, D. L., WESTON, P. G. & HIXON, J. E. (1997) Influence of estradiol and progesterone withdrawal on the secretion of and the temporal correlation between pulses of oxytocin and prostaglandin F-2 alpha in ewes. *Biology of Reproduction*, 56, 1228-1238.
- WALLACE, W. H. B., SHALET, S. M., HENRY, J. H., MORRIS, P. H. & GATTAMANENI, H. R. (1989) Ovarian failure following abdominal irradiation in childhood: the radiosensitivity of the human oocyte. *British Journal of Radiology*, 62, 992-998.
- WALMSLEY, E., POLLARD, J., PLANTE, C., SONGSASEN, N. AND BUCKRELL, B. (1996) The effects of age and repeated aspirations on the number of oocytes collected from pre-pubertal Arcott lambs. In: Thompson JG (eds), *Techniques for gamete Manipulation and Storage*. ICAR Proceedings, Elsevier Hamilton, New Zealand.
- WALMSLEY, S. E., BUCKRELL, B. C., BUSCHBECK, C., RUMPH, N. & POLLARD, J. W. (2004) Rate of abnormalities in lambs from in vitro produced embryos transferred on Day 2 compared with Day 6 postfertilization. *Theriogenology*, 62, 195-206.
- WANG, S., LIU, Y., HOLYOAK, G. R. & BUNCH, T. D. (1997) The effects of bovine serum albumin and fetal bovine serum on the development of pre- and postcleavage-stage bovine embryos cultured in modified CR2 and M199 media. *Animal Reproduction Science*, 48, 37-45.
- WANI, N. A., WANI, G. M., KHAN, M. Z. & SALAHUDIN, S. (2000) Effect of oocyte harvesting techniques on in vitro maturation and in vitro fertilization in sheep. *Small Ruminant Research*, 36, 63-67.
- WANKOWSKA, M., MISZTAL, T., ROMANOWICZ, K., WOJCIK-GLADYSZ, A. & POLKOWSKA, J. (2007) The interapituitary endocrine events during maturation and timing in the female sheep. *Animal Reproduction Science*.
- WARD, W., R. (1980) The breeding season and oestrous cycle. In: *Current Therapy. Theriogenology*. (ed. D. A. Morrow). , pp. 889-891. W. B. Saunders & Co., Philadelphia.
- WARZYCH, E., PEIPPO, J., SZYDŁOWSKIA, M. & LECHNIAKA, D. (2007) Supplements to in vitro maturation media affect the production of bovine blastocysts and their

- apoptotic index but not the proportions of matured and apoptotic oocytes. *Animal Reproduction Science*, 97, 334-343.
- WASSARMAN, P. M. & ALBERTINI, D. F. (1994) The Mammalian Ovum. IN KNOBIL, E. & NEILL, J. D. (Eds.) *The Physiology of Reproduction*. Second ed. New York, Raven Press, Ltd.
- WATHES, D. C., GULDENAAR, S. E. F., SWANN, R. W., WEBB, R., PORTER, D. G. & PICKERING, B. T. (1986) A Combined Radioimmunoassay and Immunocytochemical Study of Ovarian Oxytocin Production During the Perioovulatory Period in the Ewe. *Journal of Reproduction and Fertility*, 78, 167-183.
- WATHES, D. C. & LAMMING, G. E. (1995) The oxytocin receptor, luteolysis and the maintenance of pregnancy. *J Reprod Fertil Suppl*, 49, 53-67.
- WATSON, A. J., WATSON, P. H., WARNES, D., WALKER, S. K., ARMSTRONG, D. T. & SEAMARK, R. F. (1994) Preimplantation development of in-vitro-matured and in-vitro-fertilized ovine zygotes - comparison between coculture on oviduct epithelial-cell monolayers and culture under low-oxygen atmosphere. *Biology of Reproduction*, 50, 715-724.
- WEBB, R. & ENGLAND, B. G. (1982) Relationship between Lh receptor concentrations in thecal and granulosa-cells and in vivo and in vitro steroid-secretion by ovine follicles during the preovulatory period. *Journal of Reproduction And Fertility*, 66, 169-&.
- WEBB, R. & GAULD, I. K. (1987) *Endocrine control of follicular growth in sheep*. In *Follicular Growth and Ovulation Rate in Farm Animals*. Eds J. F. Roche & D. O'Callaghan. Dordrecht., Martinus Nijhoff Publishers.
- WEBB, R., GAULD, I. K. & DRIANCOURT, M. A. (1989) Morphological and functional characterization of large antral follicles in three breeds of sheep with different ovulation rates.
- WEBB, R., GOSDEN, R. G., TELFER, E. E. & MOOR, R. M. (1999) Factors affecting folliculogenesis in ruminants. *Animal Science*, 68, 257-284.
- WHEATON, J. E., CARLSON, K. M., WINDELS, H. F. & JOHNSTON, L. J. (1993) Cidr - a new progesterone-releasing intravaginal device for induction of estrus and cycle control in sheep and goats. *Animal Reproduction Science*, 33, 127-141.
- WILMUT, I., SALES, AND ASHWORTH, C. J. (1986) Maternal and embryonic factors associated with prenatal loss in mammals. *J. Reprod. Fertil.*, 76, 851-864.
- WILTBANK, J. N. & CASIDA, L. E. (1956) Alteration of ovarian by hysterectomy. *J. Anim Sci*, 15, 134-140.
- WILTBANK, M. C., DISKIN, M. G., FLORES, J. A. & NISWENDER, G. D. (1990) Regulation of the corpus luteum by protein kinase C. II. Inhibition of lipoprotein-stimulated steroidogenesis by prostaglandin F₂ alpha. *Biol Reprod*, 42, 239-245.
- WILTBANK, M. C., DISKIN, M. G. & NISWENDER, G. D. (1991) Differential actions of 2nd messenger systems in the corpus-luteum. *Journal of Reproduction And Fertility Supplement*, 65-75.
- WOODFILL, C. J. I., WAYNE, N. L., MOENTER, S. M. & KARSCH, F. J. (1994) Photoperiodic Synchronization Of A Circannual Reproductive Rhythm In Sheep - Identification Of Season-Specific Time Cues. *Biology Of Reproduction*, 50, 965-976.
- YANAGISHITA, M. & HASCALL, V. C. (1979) Isolation and characterisation of proteoglycans. *Journal Biology Chemistry*, 254, 911-920.
- YELLON, S. M., FOSTER, D. L., LONGO, L. D. & SUTTIE, J. M. (1992) Ontogeny of the pineal melatonin rhythm and implications for reproductive development in domestic ruminants. *Animal Reproduction Science*, 30, 91-112.

- YING, S.-Y. & GREEP, R. O. (1971) Prevention of aminoglutethimide phosphate (AGP) block of ovulation in PMS-treated immature rats. *Proceedings of the Society for Experimental Biology and Medicine*, 136, 916-919.
- YOSHIMURA, Y., NAKAMURA, Y., YAMADA, H., ANDO, M., UBUKATA, Y., ODA, T. & SUZUKI, M. (1991) Possible contribution of prolactin in the process of ovulation and oocyte maturation. *Horm Res*, 35 Suppl 1, 22-32.
- YOUNIS, A. I., BRACKETT, B. G. & FAYRERHOSKEN, R. A. (1989) Influence of serum and hormones on bovine oocyte maturation and fertilization invitro. *Gamete Research*, 23, 189-201.
- YUE, G. H., XIE, C. X. & CHENG, R. H. (1996) Development of reproductive system and levels of sexual hormones in blood plasma in chinese hu sheep ewe lambs. *Reprod in Domestic Animals*, 31, 725-728.
- ZHANG, J., WESTON, P. G. & HIXON, J. E. (1992) Role of progesterone and estradiol In the regulation of Uterine oxytocin receptors In Ewes. *Journal of Reproduction and Fertility*, 94, 395-404.
- ZIEBA, D. A., MURAWSKI, M. G. & WIERZCHOS, E. (2000) The role of luteal steroid hormones in the regulation of the estrous cycle of high fecundity Olkuska sheep. *Animal Reproduction Science*, 58, 87-98.
- ZUELKE, K. A. & BRACKETT, B. G. (1990) Luteinizing hormone-enhanced invitro maturation of bovine oocytes with and without protein supplementation. *Biology of Reproduction*, 43, 784-787.

Appendices

Appendix.2.A.Ewes information for year one

Animal No.	Breed	Weight/k g	B. Score	Animal No.	Breed	Weight/k g	B. Score
162	Walsh	39.5	2.00	234	"	35.5	1.75
41075	"	45.0	2.00	185	Speckle Black	54.5	2.00
99	"	41.5	2.00	13	face	62.0	2.75
81	"	50.5	2.00	168	Speckle	46.5	2.5.0
109	"	38.5	2.00	255	Walsh	42.0	2.75
236	Speckle	55.0	2.75	246	"	36.5	2.00
76	"	60.0	1.75	145	Speckle	46.5	2.00
48	"	54.0	2.50	1	"	54.0	2.75
132	"	55.0	3.00	144	"	56.0	2.75
131	"	59.0	2.75	226	Walsh	39.0	2.75
41075	Walsh	38.5	1.50	156	"	51.0	3.00
226	"	37.0	2.00	164	"	51.0	2.75
	Black						
16	face	36.5	2.75	155	Speckle	45.5	2.75
66	"	57.5	1.75	133	Walsh	49.5	3.00
125	"	70.5	3.00	127	"	38.5	2.75
					Black		
126	"	45.5	1.75	132	face	63.0	2.75
159	Walsh	39.5	2.00	116]	Walsh	50.0	2.00
73	"	35.0	2.00	180	Walsh	35.5	2.50
7	"	44.0	2.75	135	"	58.0	2.50
4	"	36.5	2.75	151	"	37.5	2.00
199	"	39.5	2.75	210	"	46.5	2.50
					Black		
137	"	36.5	2.75	20	face	40.0	2.75
184	Walsh	40.0	2.50	167	Walsh	45.0	2.50
14	"	43.5	2.50	216	Blackfac	51.0	2.00
99	"	44.0	3.00	41087	"	57.0	2.50
72	"	38.0	3.00	234	"	33.5	1.50
169	Blackfa	52.0	1.75	15	Speckle	38.5	2.50
224	Walsh	39.5	2.00	214	"	42.0	2.75
166	Blackfa	66.0	2.50	128	"	35.5	1.75
148	"	52.5	2.50	110	Walsh	54.5	2.00
128	"	52.5	2.00	254	"	62.0	2.75
231	Speckle	43.5	2.75	199	"	46.5	2.50
240	"	56.5	2.00	40215	"	42.0	2.75
35	"	48.5	2.50	109	"	55.0	2.75
211	Walsh	55.0	3.25	267	Speckle	60.0	1.75
203	"	37.0	2.50	233	Walsh	54.0	2.50
189	"	39.5	2.00	101	"	55.0	3.00
164	"	44.5	2.50	19	Speckle	59.0	2.75
232	"	38.5	2.00	161	Walsh	38.5	1.50
136	Speckle	40.0	2.00	222	"	37.0	2.00

Animal No.	Breed	Weight/kg	B. Score	Animal No.	Breed	Weight/kg	B. Score
134	Walsh	40.5	2.75	213	"	36.5	2.75
89	"	35.5	2.50	102	Speckle	57.5	1.75
149	Speckle	58.0	2.50	134	Speckle	70.5	3.00
217	Walsh	37.5	2.00	145	"	45.5	1.75
197	"	46.5	2.50	106	Walsh	39.5	2.00
128	"	40.0	2.75	205	"	35.0	2.00
146	Speckle	45.0	2.50	213	"	44.0	2.75
221	Speckle	51.0	2.00	267	Speckle	36.5	2.75
171	"	57.0	2.50	209	Blackfac	40.0	2.50
258	Walsh	33.5	1.50	210	Speckle	43.5	2.50
47	"	38.5	2.50	211	Walsh	44.0	3.00
250	"	42.0	2.75				

Appendix.2.A.Ewe lambs information, year one.

Animal No.	Weight/kg	B. Score	Animal No.	Weight/kg	B. Score
4806	37.5	3.00	4867	31.5	3.25
4807	42.0	3.50	4868	31.5	2.75
4808	39.0	3.50	4869	49.5	4.00
4809	36.5	3.50	4870	33.5	2.75
4810	37.0	3.00	4871	32.5	3.00
4811	39.5	3.25	4872	35.0	2.75
4812	36.0	3.50	4873	31.5	3.00
4814	39.0	3.50	4874	41.5	3.25
4815	35.5	3.50	4875	41.0	3.00
4816	30.5	3.25	4876	39.0	3.25
4817	33.5	3.25	4877	34.5	3.25
4818	35.5	3.75	4878	35.0	3.00
4819	37.0	3.25	4879	34.5	2.75
4820	31.0	3.00	4880	37.0	3.00
4821	38.5	3.25	4881	34.5	3.50
48.23	27.5	2.50	4882	32.5	3.00
48.24	30.0	2.75	4883	39.5	2.75
48.25	32.0	2.75	4884	36.5	3.00
48.26	38.0	3.50	4885	27.5	2.75
48.27	38.0	3.50	4886	37.5	2.50
48.28	35.0	3.00	4887	40.0	3.75
48.29	32.5	3.50	4888	32.0	2.75
4831	32.0	3.00	4889	34.5	3.50
4832	34.5	3.25	4890	34.5	3.25
4833	36.5	3.50	4891	36.0	3.50
4834	38.5	3.00	4892	32.5	2.50
4835	29.0	3.00	4893	33.0	3.50
4836	38.0	3.00	4894	24.0	2.50
4837	55.0	3.25	4895	35.5	2.00
4838	25.0	2.75	4896	26.5	3.00
4839	31.5	3.00	4897	30.0	3.00
4840	36.0	3.50	4898	31.5	2.75
4841	39.0	3.00	4899	32.5	3.00
4842	33.5	3.50	4900	34.0	3.00
4843	34.5	3.00	4901	33.5	3.00
4844	37.5	3.50	4902	36.5	3.00
4845	37.0	2.75	4903	36.5	3.25
4846	34.5	3.00	4904	37.0	3.00
4847	40.0	3.75	4905	37.0	3.50
4848	40.0	3.00	4906	34.0	3.00
4849	34.0	3.00	4907	28.0	2.50
4850	33.0	3.25	4908	34.0	3.00
4851	40.5	3.50	4909	36.0	3.00

Animal No.	Weight/kg	B. Score	Animal No.	Weight/kg	B. Score
4852	33.5	3.25	4910	40.0	3.25
4853	33.5	2.75	4911	36.0	3.50
4854	35.0	3.25	4912	36.0	3.50
4855	36.0	3.00	4913	37.5	3.50
4856	36.5	3.00	4914	27.0	2.75
4857	34.0	3.50	4915	35.0	3.50
4858	38.5	3.00	4916	40.0	3.00
4859	30.0	2.75	4917	33.0	3.00
4860	41.0	3.50	4918	33.0	3.00
4861	35.5	3.25	4919	30.0	3.00
4862	38.0	3.50	4920	38.0	3.25
4863	32.0	3.50	4921	39.5	3.50
4864	32.5	2.75	4922	39.0	3.00
4865	35.5	3.50	4923	38.0	3.50
4866	36.0	3.25	4924	34.5	2.75

Appendix.2.B.Ewes information for year two.

Ewe Tag	Weight	B. Score	Breed	Ewe Tag	Weight	B. score	Breed
OR 45	42.0	2.5	Walsh	G 067	34.0	1.0	Walsh
G 030	43.0	3.0	"	G 056	47.0	2.0	Speckle
G 130	47.0	3.5	"	P 248	46.5	2.0	Walsh
Y 145	48.0	3.5	"	G 042	54.5	4.0	Speckle
G 026	51.0	3.5	"	P 196	46.5	2.5	Walsh
G 174	51.5	3.5	"	Y 147	35.5	2.0	"
P 243	47.5	3.0	"	P 110	37.5	2.0	"
P 254	41.0	1.0	"	Y 8157	45.5	3.0	"
P 166	39.0	2.0	"	M 002	45.5	2.0	"
P 101	38.5	2.0	"	G 230	38.5	2.0	"
G 093	43.5	2.5	"	P 154	47.0	2.5	"
M 005	40.5	2.0	"	Y 151	42.0	2.0	"
G 055	50.0	2.5	"	G 212	41.0	2.5	"
P 201	45.0	3.0	"	P 179	48.0	2.5	"
G 124	61.5	4.0	"	M 013	40.0	2.5	"
G 146	45.0	2.0	Speckle	G 158	43.5	3.0	"
OR 44	41.5	2.5	Walsh	M 002	40.5	2.0	"
G 97	51.0	3.0	"	OR 44	47.5	2.5	"
G 200	46.5	3.0	"	P 220	42.5	2.5	"
G 254	48.5	2.5	"	GZ 15	36.0	2.0	"
G 235	36.0	2.5	"	M 001	39.0	2.0	"
G 106	42.5	2.5	"	G 082	47.0	3.0	"
G 192	39.0	2.0	"	P 163	40.5	2.0	"
Y 157	43.5	2.5	"	P 208	45.5	3.0	"
G 044	39.0	2.0	"	P 233	55.5	3.0	Scotsh
G 054	52.0	2.5	Speckle	G 111	66.5	3.0	"
M 005	44.5	2.5	Walsh	M 053	42.5	2.0	Walsh
P 227	44.5	3.0	"	M 002	41.0	2.5	"
Y 158	44.5	3.0	"	P 138	48.0	3.5	"
G 141	44.0	2.5	"	G 120	39.5	2.5	"
G 210	44.0	2.5	"	M 001	47.5	2.0	"
P 192	59.5	3.0	Speckle	G 191	48.0	2.5	Walsh
M 005	55.0	3.0	Walsh	P 253	37.0	2.0	"
P 100	53.5	2.5	"	G 101	50.5	3.5	"
G 119	61.0	3.0	Speckle	G 238	43.0	2.0	"
P 249	46.5	2.0	Walsh	P 244	48.5	2.5	"
G 080	65.0	3.5	Speckle	P 206	55.0	3.0	"
P 141	66.0	3.5	"	G 234	46.5	2.5	"
G 086	42.5	2.5	Walsh	165	39.5	2.0	"
Y 141	39.0	2.0	"	83	51.5	3.0	Speckle
G 122	51.0	3.5	Speckle	G 168	72.0	3.0	Scotsh
P 153	60.5	4.0	Walsh	G 218	40.0	2.0	Walsh
P 241	42.0	2.0	"	P 108	39.5	2.0	"
P 247	45.5	3.0	"	G 090	62.5	3.0	Scotsh

Ewe Tag	Weight	B. Score	Breed	Ewe Tag	Weight	B. score	Breed
P 090	37.5	1.5	"	M 026	49.0	2.0	Specal
G 030	46.0	3.0	Walsh	G 070	49.0	3.5	Walsh
G 096	44.0	2.5	"	G 197	38.0	3.5	"
P 046	53.0	3.5	Speckle	G 173	44.0	2.0	Speckle
P 167	60.5	3.5	"	R 124	52.5	3.0	Walsh
M 0039	42.0	2.5	Walsh	G 108	40.0	2.0	"
P 040	38.5	2.5	"	P 181	44.5	2.5	"
G 043	43.0	2.5	"	P 203	57.5	2.0	Speckle
P 252	44.0	2.5	"	G 250	54.0	3.0	Walsh
P 260	49.0	3.0	"	P 151	38.5	2.0	"
OR 44	46.0	2.0	"	Y 154	49.5	3.5	"
P 214	56.0	3.0	Speckle	OR 44	49.5	3.5	"
P 076	37.0	2.0	Walsh	Y 141	49.0	2.5	"
M 010	44.5	2.0	"	C 263	58.0	2.5	<i>Liey tesar</i>
P 183	41.5	2.0	"	C 364	64.0	3.0	" "

Appendix.2.B Lambs information for year two.

Lamb Tag	Weight	B. score	Lamb Tag	Weight	B. score	Lamb Tag	Weight	B. score
3291	37.0	2.75	4234	36.0	2.75	4268	30.5	2.50
4201	36.5	2.00	4235	30.0	2.50	4269	30.5	2.25
4202	34.5	2.25	4236	34.0	2.75	4270	31.0	2.50
4203	35.0	2.25	4237	38.5	3.50	4271	33.5	1.75
4204	35.5	2.50	4238	35.0	2.50	4272	32.5	2.00
4205	37.5	2.50	4239	34.0	3.50	4273	28.5	2.75
4206	36.0	2.75	4240	34.0	3.75	4274	33.5	3.00
4207	39.0	3.25	4241	37.5	3.50	4275	27.0	2.25
4208	31.5	2.75	4242	35.5	2.00	4276	37.0	2.00
4209	32.5	2.00	4243	33.0	1.50	4277	32.5	2.00
4210	28.5	2.00	4244	38.0	2.25	4278	27.5	2.25
4211	31.5	3.00	4245	27.5	2.50	4279	29.0	2.00
4212	36.0	3.00	4246	34.5	2.50	4280	34.5	2.25
4213	34.5	2.25	4247	34.5	2.50	4281	35.0	2.00
4214	31.5	3.00	4248	33.0	2.75	4282	29.5	2.00
4215	30.5	2.00	4249	37.5	2.50	4283	30.0	2.25
4216	27.5	2.25	4250	29.5	2.50	4284	30.5	2.50
4217	36.0	2.75	4251	40.5	2.75	4285	39.0	2.75
4218	26.0	2.00	4252	30.0	2.25	4286	38.5	3.00
4219	38.0	2.50	4253	32.5	3.00	4287	39.5	3.50
4220	36.5	2.50	4254	35.0	2.75	4288	33.0	2.50
4221	31.5	1.75	4255	38.5	3.50	4289	32.5	2.25
4222	27.0	2.50	4256	35.0	3.00	4290	31.0	2.00
4223	35.5	3.50	4257	35.0	2.50	4291	37.5	2.75
4224	31.5	2.00	4258	34.0	2.75	4292	31.5	2.00
4225	33.0	2.75	4259	33.5	2.50	4293	34.5	3.50
4226	33.0	3.00	4260	38.0	3.00	4294	33.5	2.00
4227	34.0	3.50	4261	35.0	2.50	4295	34.5	2.75
4228	30.5	2.25	4262	33.0	2.50	4296	37.0	1.75
4229	39.5	2.00	4263	32.5	1.75	4297	31.0	2.50
4230	38.5	2.75	4264	29.0	2.00	4298	33.5	3.75
4231	26.0	2.00	4265	32.5	2.75	4299	29.0	2.75
4232	32.0	2.25	4266	36.5	2.00			
4233	36.0	2.75	4267	25.5	1.50			

Appendix.2.C.Ewes information for third year.

Ewe Tag	Weight	Con. Score	Breed	Notes
G820	33.5	1.5	Walsh	
M0046	40.0	2.0	"	
R057	45.0	3.5	"	
R103	44.0	3.0	"	
G830	39.5	2.0	"	
Z255	51.5	4.0	"	
G821	37.0	1.5	"	
R018	47.5	2.5	"	
Z116	38.0	2.5	"	G201
G99	59.5	4.0	"	
G051	47.5	2.0	Speckle	
G252	40.5	2.5	Walsh	
G005	41.0	4.0	"	
R043	40.5	3.0	"	G1277
R230	42.5	2.0	"	
R155	44.0	4.0	"	
G115	59.5	3.5	"	2275
G237	39.0	2.5	"	
CJDW	32.0	1.0	Speckle	63
M822	36.0	2.5	"	Z2117
R070	31.5	1.0	"	
R111	47.5	3.0	"	
G822	40.5	2.5	"	
R079	60.0	4.5	"	247
M003	40.5	3.0	"	
R035	50.5	4.0	"	Z245
Y0001562	40.0	1.5	"	
G1275	39.0	2.0	"	
G036	34.5	2.0	"	
G085	42.5	2.5	"	
R106	53.5	4.5	"	
R257	40.0	2.5	"	
M0031	46.5	3.5	"	
G1406	32.5	1.0	"	
G824	41.4	2.5	"	
G069	46.0	2.5	"	
G018	43.0	4.0	"	
blow22	39.5	3.5	"	000blow22
Y0001418	50.0	3.0	"	
M0045	44.0	2.5	"	
Penk405	48.5	2.0	Speckle	
Y0001566	47.5	3.0	Walsh	
G1412	45.5	2.0	"	
G1281	45.0	1.5	"	

Ewe Tag	Weight	Con. Score	Breed	Notes
R041	38.5	2.5	"	
G1404	42.0	3.0	"	
R126	40.5	2.5	"	
G041	41.0	2.0	"	
G182	44.5	2.0	"	
Y0001486	40.0	1.5	"	
Y000817	47.0	2.0	Speckle	
YC8157	44.5	2.5	Walsh	
G1290	50.5	3.0	"	
G136	42.0	2.0	"	
Y0001536	39.5	2.5	"	
3744237	41.0	2.5	"	green neck
M009	47.5	2.5	"	
Y0001461	46.5	3.5	"	
G0222	41.5	2.5	"	
Y06	47.5	2.0	"	R47
R180	48.5	2.5	"	1998
G1430	41.5	3.0	"	
G138	42.0	3.0	"	
P428	36.5	1.5	Speckle	
R090	35.0	1.5	Walsh	YC8157
M0006	46.0	2.5	"	green neck
R096	38.5	2.5	"	
M0041	39.0	2.0	"	
M0020	39.0	1.5	"	
M0059	42.0	2.5	"	
Y0001417	36.0	2.0	"	
G034	52.5	3.0	"	
R113	33.0	1.5	"	
R048	41.5	2.5	"	
without	41.0	1.5	Speckle	green neck
M0028	48.0	3.0	Walsh	
R006	51.5	3.0	"	GZ127
Z273	43.0	4.0	"	
M0014	46.5	3.0	"	
M0029	45.5	2.0	"	
R050	42.0	2.5	"	
G151	43.5	3.0	"	2292
M0001	43.5	3.0	"	
G790	43.0	2.5	"	
Z201	39.0	2.5	"	
Y0001428	45.0	2.5	"	
Y0001429	40.0	3.5	"	
R115	38.5	2.0	"	2276
Y0001544	47.0	2.5	"	
M0064	45.5	3.0	"	

Ewe Tag	Weight	Con. Score	Breed	Notes
G243	37.5	1.5	"	G1267
R017	50.5	3.0	"	
G1416	41.0	2.5	"	
Z256	49.5	4.0	"	
G110	41.0	3.5	"	
Y0001440	52.0	2.5	"	14401
R020	42.0	2.5	"	
G1409	35.5	1.5	"	
G1422	38.5	2.5	"	
R056	49.0	3.0	"	

Appendix.2.C.Lambs information of third year.

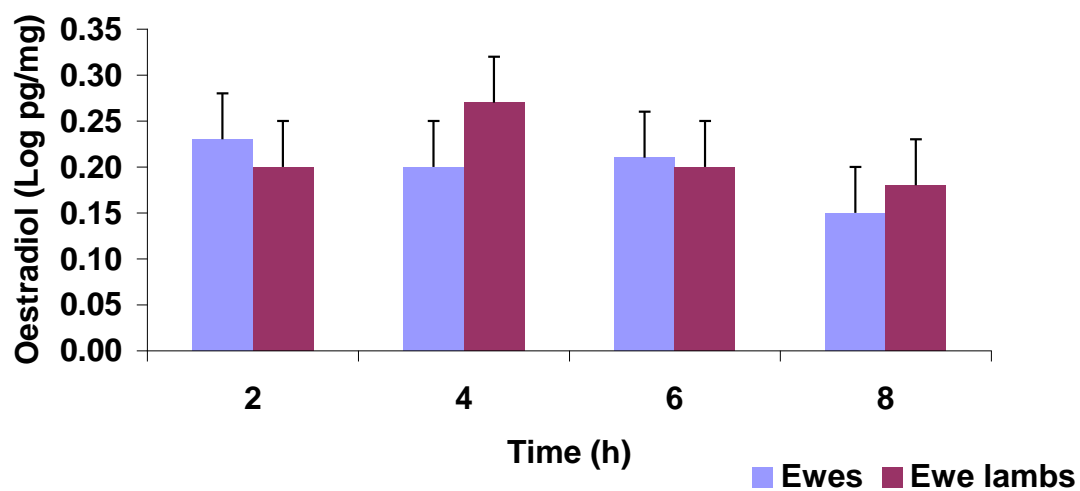
Lamb Tag	Weight	Con. Score	Lamb Tag	Weight	Con. Score
6001	41.8	3.0	6050	32.0	3.0
6002	43.9	4.0	6051	35.0	3.0
6003	31.4	2.5	6052	36.0	3.0
6004	36.2	3.0	6053	46.3	3.0
6005	41.0	3.0	6054	35.2	3.0
6006	35.6	4.0	6055	37.6	2.0
6007	43.0	4.0	6056	43.5	2.5
6008	32.2	3.0	6057	35.9	3.0
6009	44.6	4.0	6058	35.9	3.5
6010	39.5	3.0	6059	36.0	3.0
6011	31.4	3.0	6060	44.8	3.5
6012	34.7	3.5	6061	38.1	3.0
6013	39.2	4.0	6062	33.5	3.5
6014	33.9	2.5	6063	40.0	3.0
6015	33.5	4.0	6064	35.5	3.0
6016	37.2	3.0	6065	31.4	3.0
6017	37.6	2.5	6066	41.2	3.0
6018	42.0	3.5	6067	38.9	3.0
6019	34.9	3.0	6068	35.2	3.0
6020	34.5	3.0	6069	35.4	3.0
6021	37.9	2.5	6070	42.8	3.0
6022	36.9	2.5	6071	32.4	3.0
6023	34.0	2.5	6072	37.1	2.5
6024	42.9	3.0	6073	37.6	3.0
6025	37.1	3.5	6074	37.6	3.0
6026	40.3	3.5	6075	38.1	3.5
6027	41.6	3.5	6076	40.7	3.0
6028	43.2	4.0	6077	33.1	3.0
6029	42.5	2.5	6078	39.1	2.5
6030	33.7	3.0	6079	38.8	3.0
6031	40.2	2.5	6080	42.6	2.5
6032	34.3	3.0	6081	43.6	3.0
6033	32.6	2.5	6082	44.2	2.5
6034	35.6	3.0	6083	40.6	3.5
6035	38.1	3.5	6084	32.8	3.0
6036	45.6	3.0	6085	35.8	3.0
6037	40.0	4.0	6086	39.1	4.0
6038	37.4	3.0	6087	37.5	3.0
6039	34.1	3.0	6088	36.8	3.5
6040	35.0	4.0	6089	30.1	2.5
6041	39.6	3.0	6090	36.7	3.0
6042	36.6	3.0	6091	40.4	4
6043	38.6	3.5	6092	37.9	3.5
6044	37.0	2.5	6093	37.5	3.5

Lamb Tag	Weight	Con. Score	Lamb Tag	Weight	Con. Score
6044	37.0	2.5	6093	37.5	3.5
6045	37.5	2.5	6094	37.2	3.5
6046	42.0	3.5	6095	36.8	3.0
6047	33.7	3.0	6096	37.3	3.0
6048	37.0	3.5	6097	33.7	3.5
6049	37.1	2.5	6098	36.9	3.5

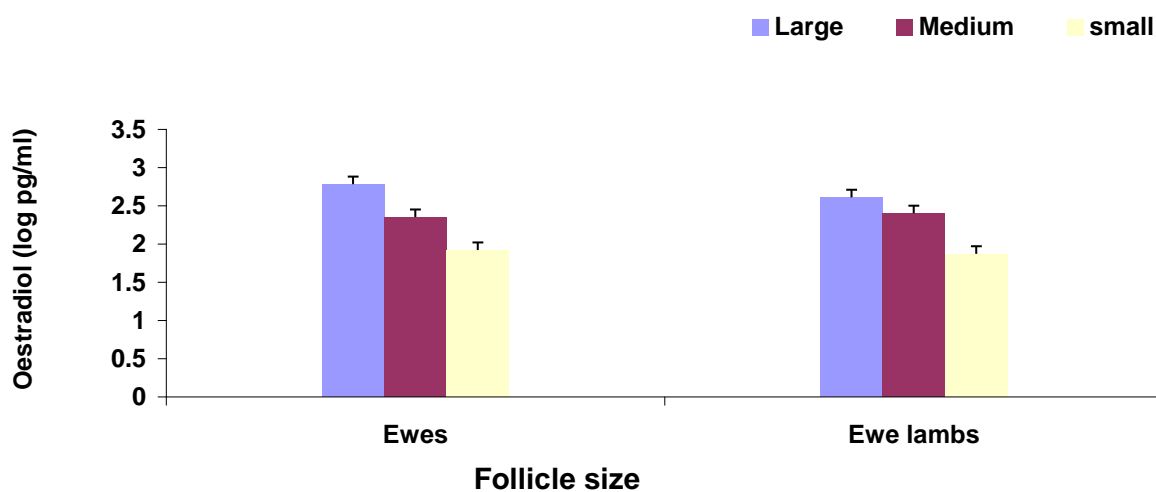
Appendix.5.1 sheep information of in vivo experimental

Ovagen Group	Animal No.	Body W. (Kg)	C. Score
lamb 1	6021	36.0	3.50
lamb 2	6023	34.5	3.50
lamb 3	6027	43.0	3.50
lamb 4	6031	38.0	3.50
lamb 5	6054	45.5	3.50
Ewe 1	480339	58.5	2.75
Ewe 2	401761	67.5	3.00
Ewe 3	480561	67.5	2.75
Ewe 4	491767	66.5	2.75
Ewe 5	401529	59.5	2.50

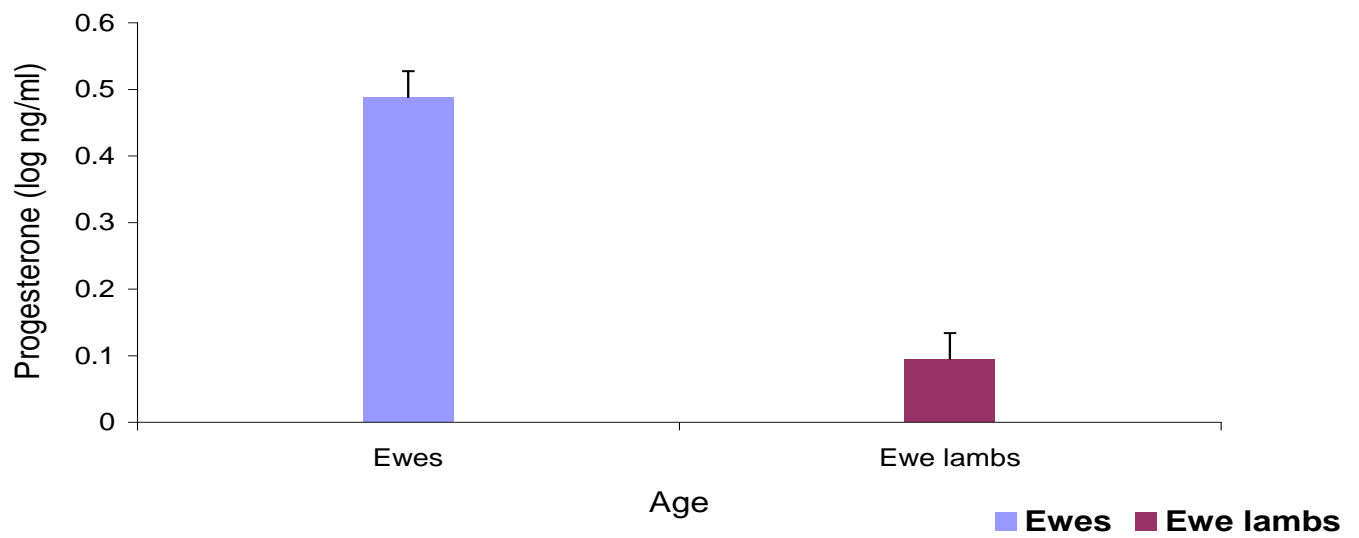
Ova + hCG group	Animal No.	Body W. (Kg)	C. Score
lamb 1	6025	39.0	3.50
lamb 2	6057	47.5	3.50
lamb 3	6065	37.50	3.50
lamb 4	6066	35.0	3.50
lamb 5	6092	43.5	3.50
Ewe 1	483377	76.5	2.75
Ewe 2	161	82.5	3.00
Ewe 3	483437	66.5	2.75
Ewe 4	493477	78.5	2.75
Ewe 5	3203	76.5	2.50



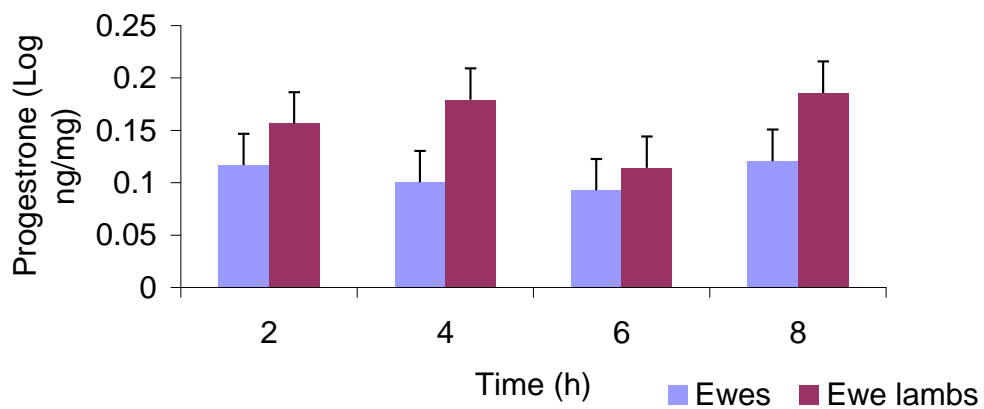
Appendix.3.1 Effect of incubation time on mean oestradiol concentrations in ewe and ewe lamb half follicles cultured in TCM-199 (SED = 0.05).



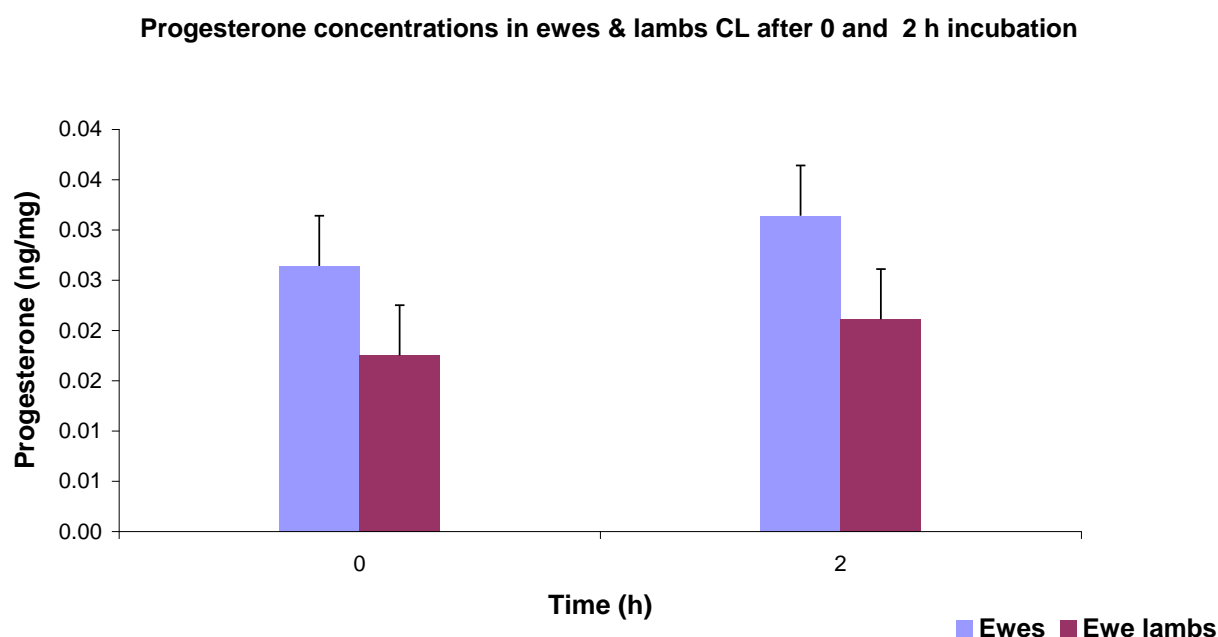
Appendix 3: 2 The effect of FCS on mean oestradiol concentration in ewe and ewe lamb follicles media (SED = 0.1).



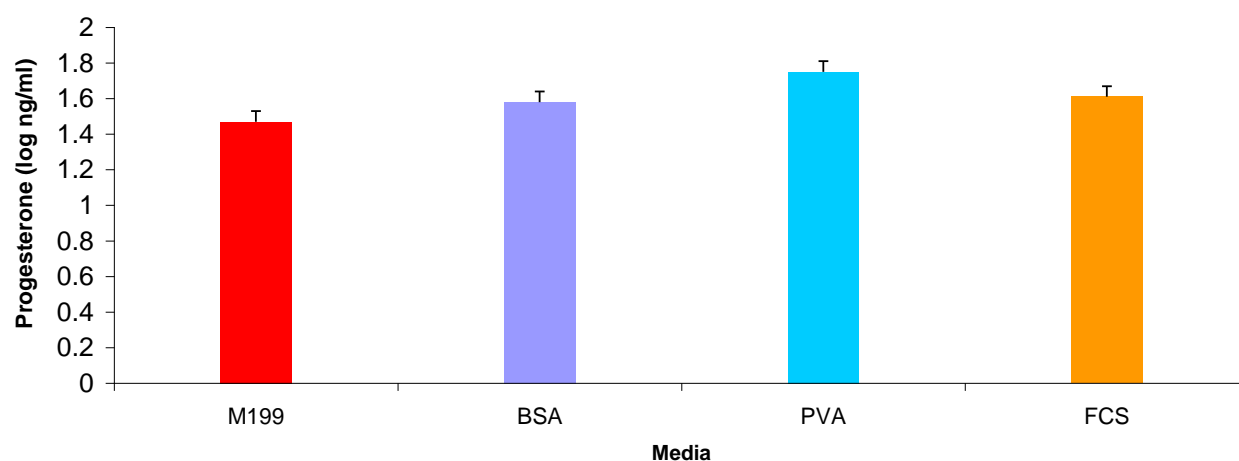
Appendix.3.3 Effect of follicle size on mean progesterone concentrations in ewe and ewe lamb follicles media after 24 h of incubation (SED = 0.04).



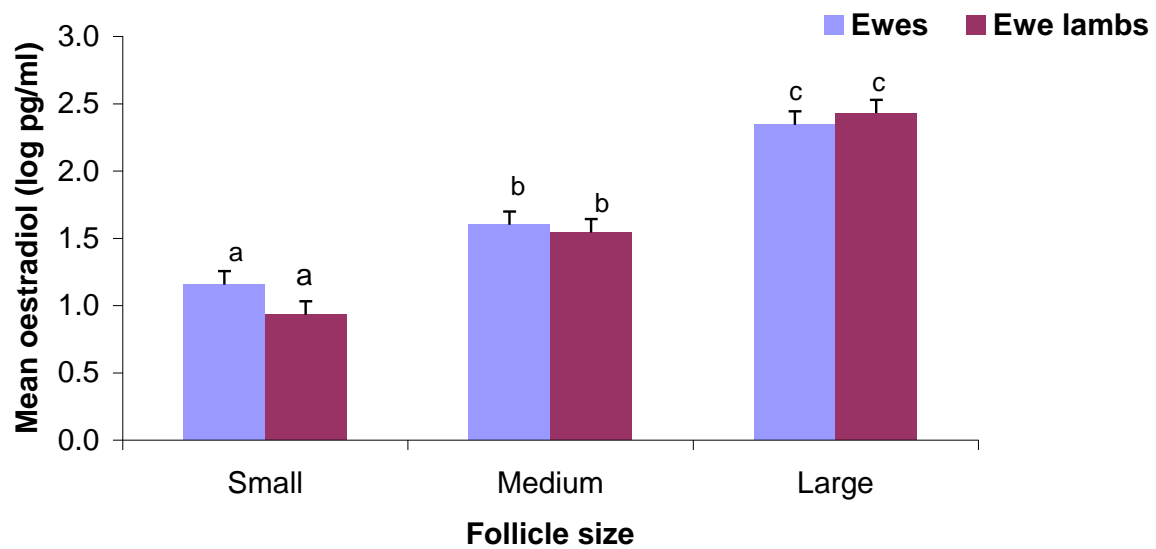
Appendix.3.4 Effect of animal age on the mean progesterone concentrations in ewes and ewe lambs follicles media cultured for 24 h in TCM-199. (SED = 0.04).



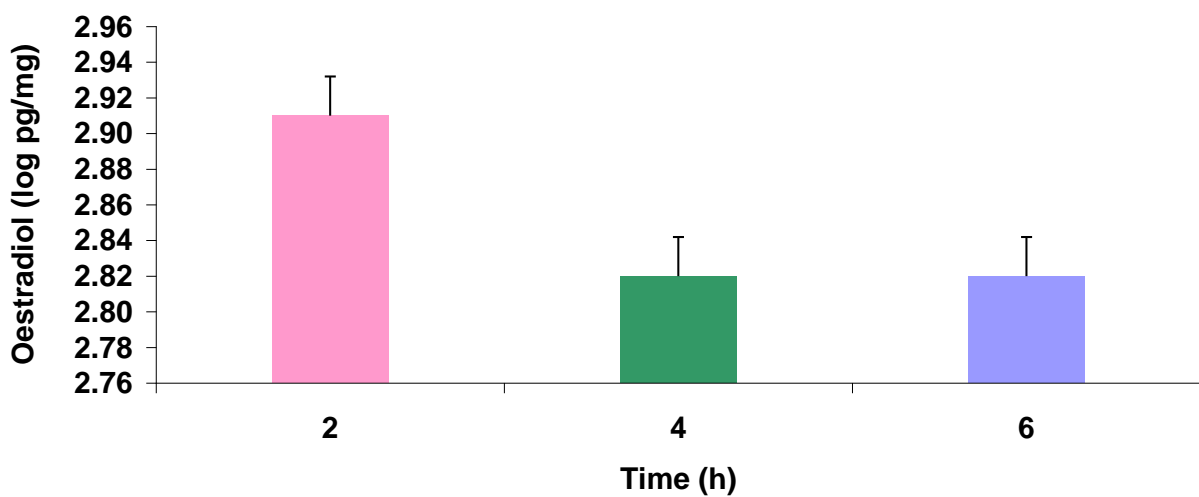
Appendix.3.5 The effect of incubation time on mean progesterone in ewe and ewe lamb CL cultured for 0 h and 2 h (SED= 0.01).



Appendix.3.6 The effect of media type on the mean progesterone concentrations in ewe and lamb CL media cultured in different media for 24 h (SED = 0.06).

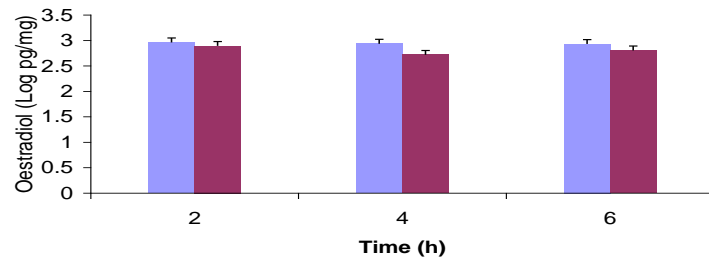


Appendix.4.1 The effect of gonadotrophin on age and follicle size on the mean oestradiol secreted from ewe and ewe lamb cultured follicles with gonadotrophin for different time (SED =0.1).

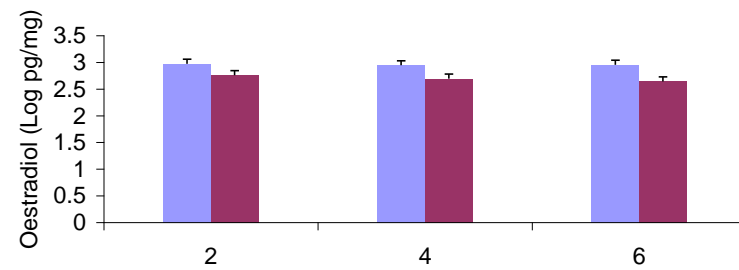


Appendix 4.2 Effect of incubation time on the mean oestradiol secreted from ewe and ewe lamb cultured follicles for different times in different concentrations of FSH/LH (SED = 0.02).

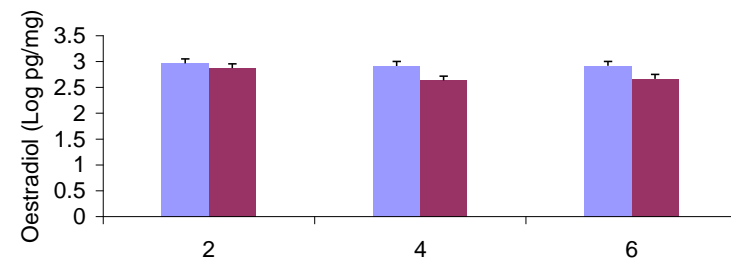
A 0/0 FSH/LH



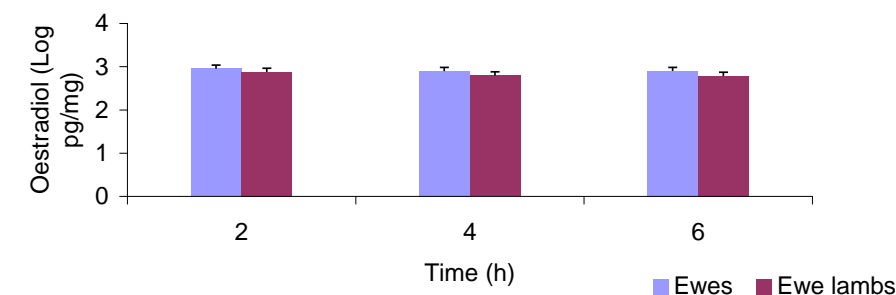
B 0.005/0.05 g/ml FSH/LH



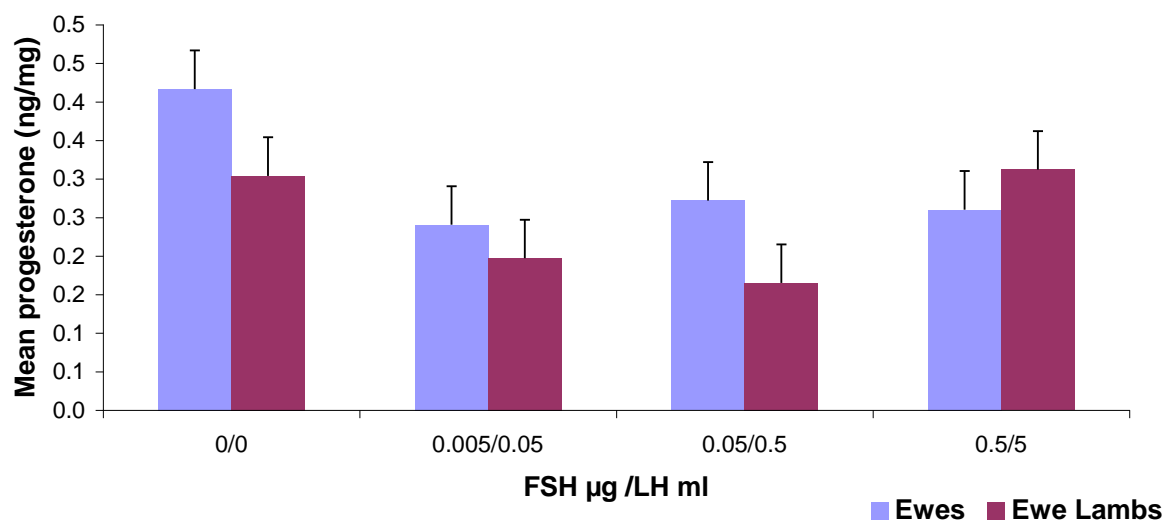
C 0.005/0.5 g/ml FSH/LH



D 0.5/5 g/ml FSH/LH

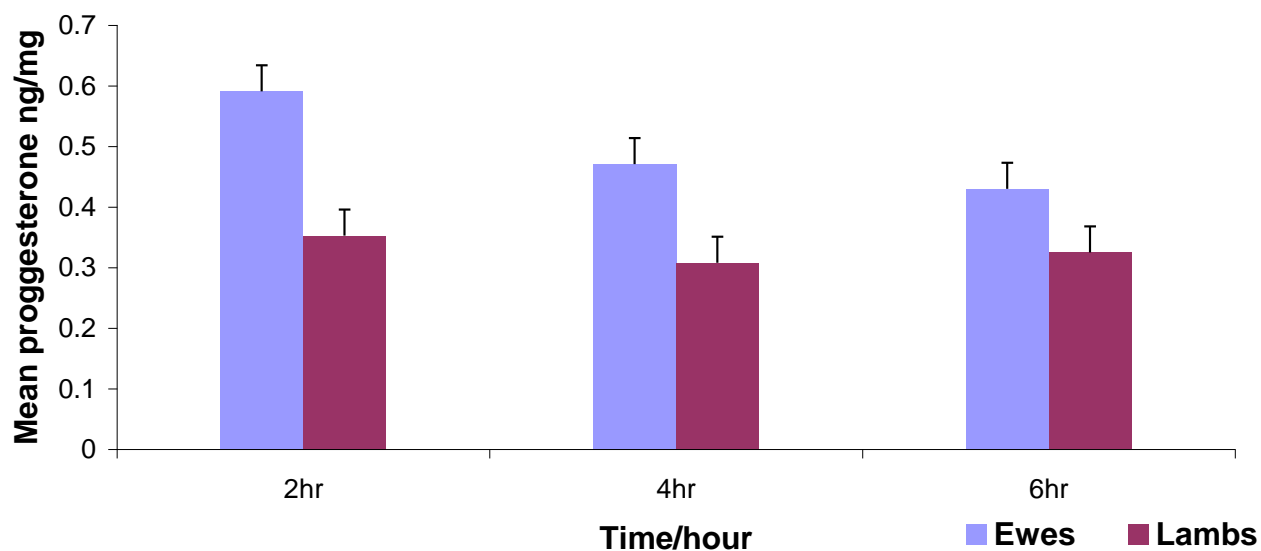


Appendix 4.3 Effect of gonadotrophin hormone on the mean progesterone secreted from ewes and ewe lambs $\frac{1}{4}$ follicles cultured in FSH/LH (SED = 0.086).

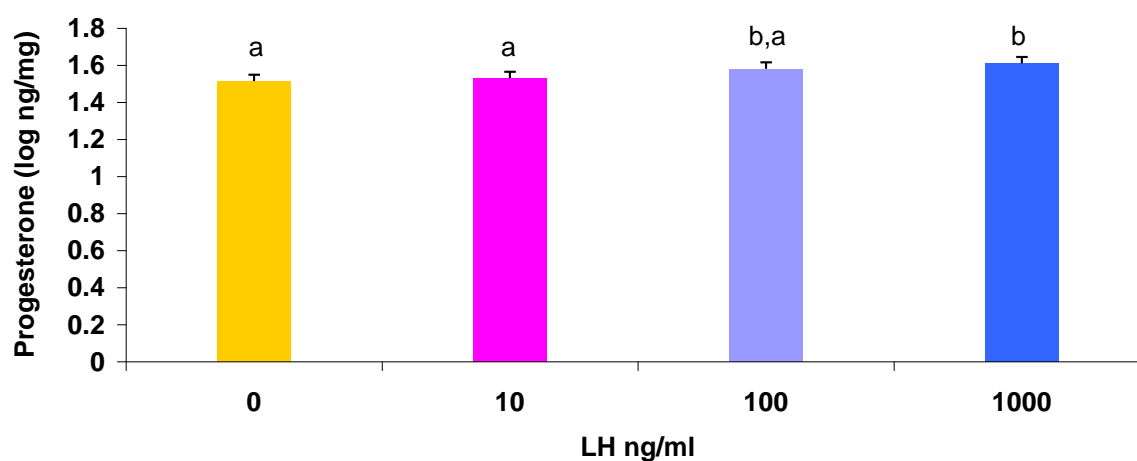


Appendix.4.4 Effect of gonadotrophin hormone on the mean progesterone secreted from ewes and ewe lambs $\frac{1}{4}$ follicles cultured in FSH/LH (SED = 0.05).

Progesterone concentrations in 1/4 ewes & lambs follicles media cultured in hCG

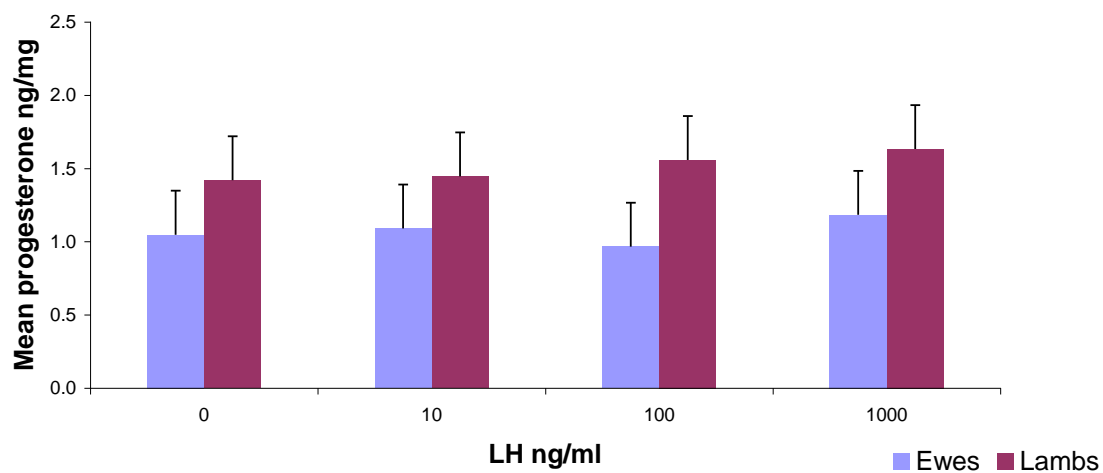


Appendix.4.5 Effect of hCG concentrations on the mean progesterone secreted from ewe and ewe lamb 1/4 follicles cultured in different amount of hCG (SED = 0.04).



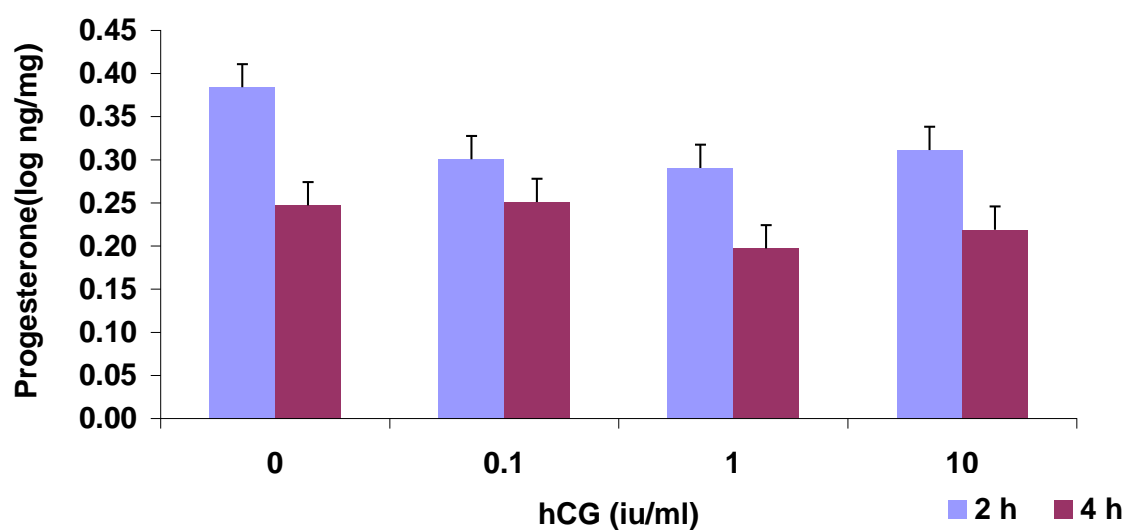
Appendix.4.6 Effect of dilution on the mean progesterone secreted from ewes and ewe lambs CL tissue (SED = 0.035).

Progesterone concentration in ewes & lambs CL media cultured in different LH concentrations after 6hrs incubation

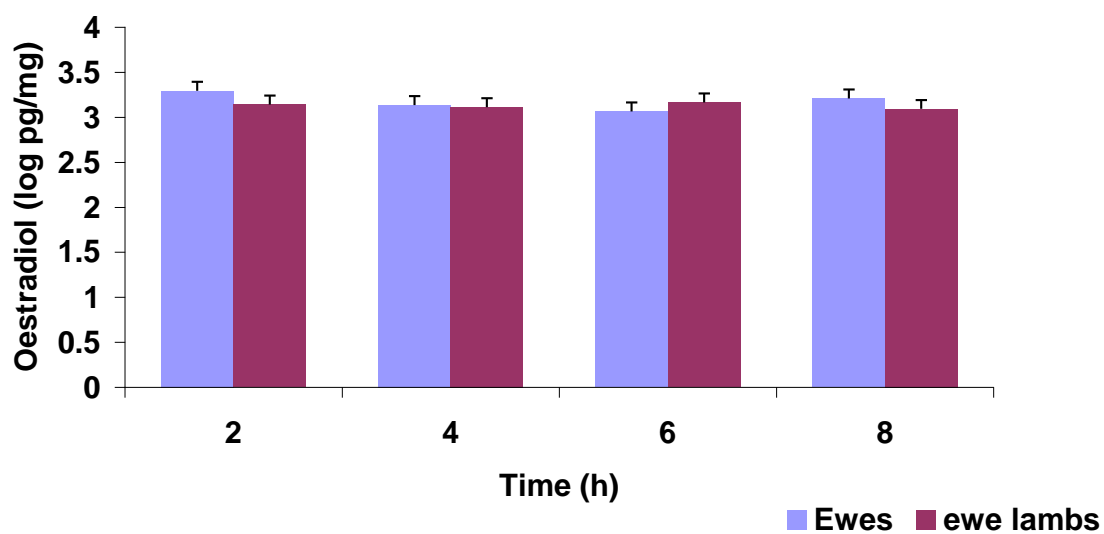


Appendix.4.7 Effect of dilution on the mean progesterone secreted from ewes and ewe lambs CL tissue (SED = 0.3).

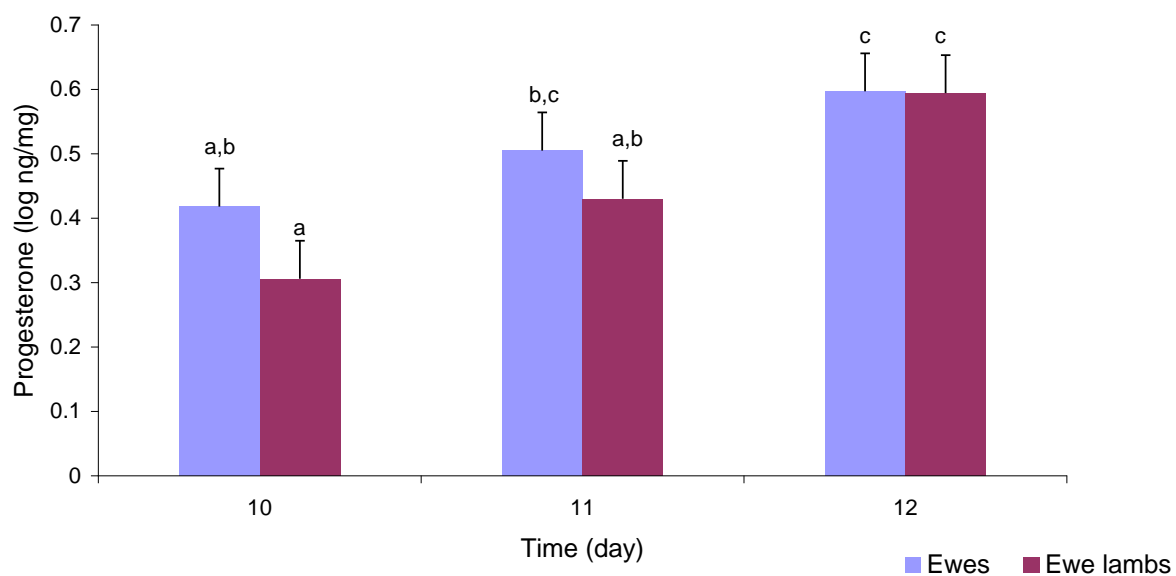
Progesterone concentrations in ewes & lambs cl cultured in hCG



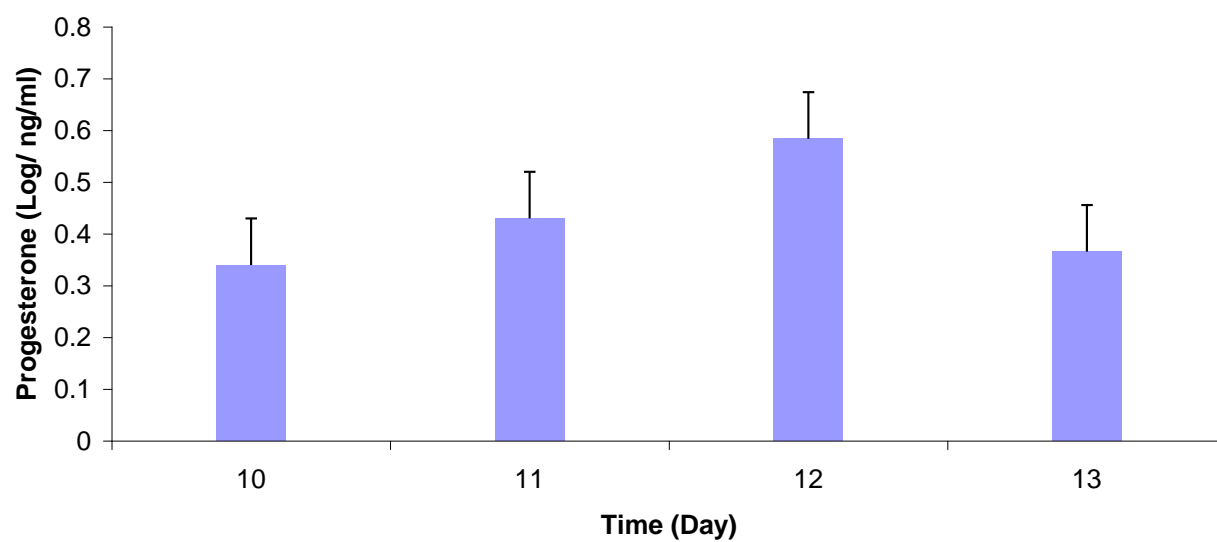
Appendix.4.8 Effect of hCG concentrations on the mean progesterone in ewe and ewe lamb CL media cultured for different time of incubation (SED = 0.27).



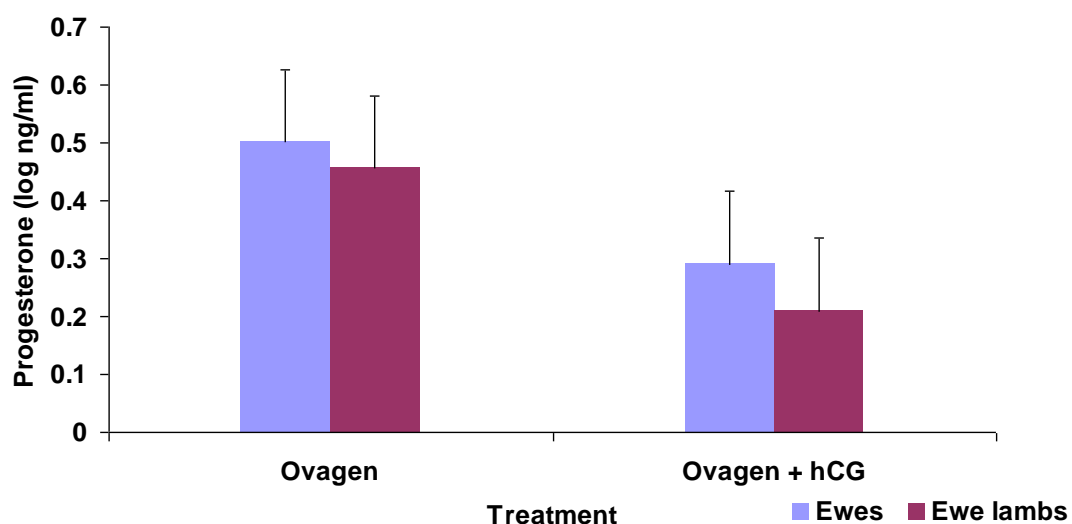
Appendix.5.1 Effect of length of incubation on mean media oestradiol secreted from ewe and ewe lamb medium and large follicles treated with ovagen (SED = 0.1).



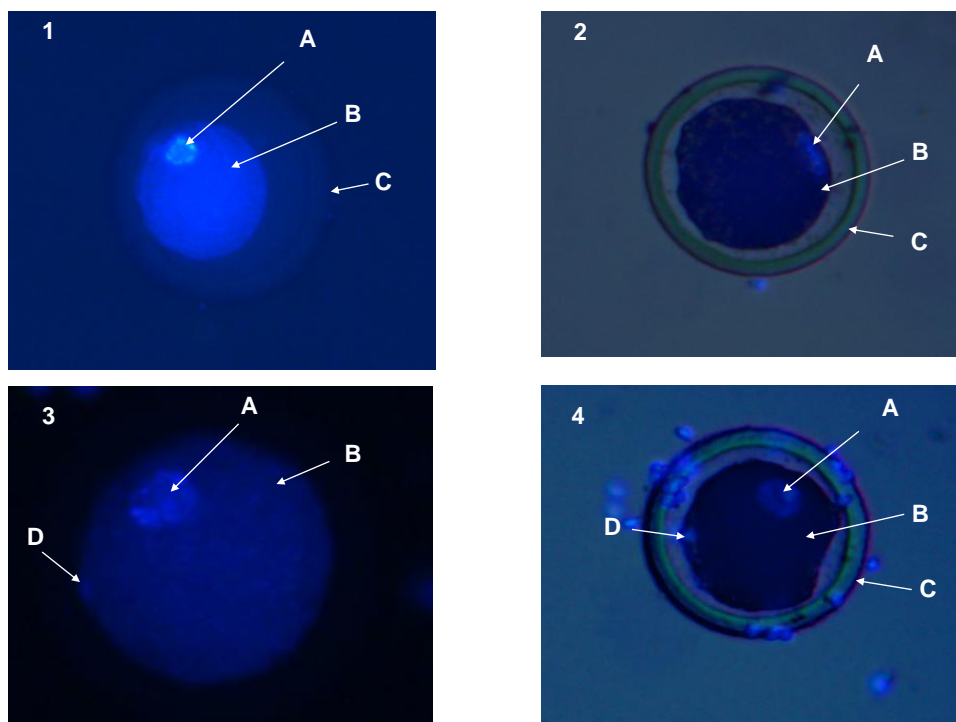
Appendix .5.2 Effect of time and treatment on mean progesterone concentration in blood samples from ewes and ewe lambs treated with ovagen (SED = 0.059).



Appendix .5.3 Effect of time and treatment on mean progesterone concentration in blood samples from ewes and ewe lambs treated with ovagen plus hCG (SED = 0.09).



Appendix .5.4 Effect animal age and treatments on mean progesterone produced from ewes and ewe lambs blood samples (SED = 0.13)



Appendix .6.1 Ewe and ewe lamb oocytes florescent images showed the oocyte (A); cytoplasm (B); cell membrane (C) and the polar body (D) (1, 2 and 4 on 300 x magnification and 3 on 400 x magnification).